

IOWA STATE COLLEGE  
**JOURNAL of SCIENCE**

*A Quarterly of Research*



VOL. VI  
1931-32

PUBLISHED BY THE  
EDITORIAL BOARD OF THE  
IOWA STATE COLLEGE JOURNAL OF SCIENCE

IOWA STATE COLLEGE

# JOURNAL OF SCIENCE

Published on the first day of October, January, April and July.

---

## EDITORIAL BOARD

EDITOR-IN-CHIEF, R. E. Buchanan.

ASSOCIATE EDITORS: P. E. Brown, Secretary; C. J. Drake, A. H. Fuller,  
I. E. Melhus, E. A. Benbrook, P. Mabel Nelson, V. E. Nelson.

ADVISORY EDITORS: R. E. Buchanan, Chairman; C. J. Drake, E. W. Lind-  
strom, I. E. Melhus, P. Mabel Nelson, O. R. Sweeney.

BUSINESS COMMITTEE: P. E. Brown, Chairman; A. H. Fuller, V. E. Nelson,  
Jay W. Woodrow.

---

All manuscripts submitted for publication should be addressed to R. E. Buchanan, Room 101 Science Building, Iowa State College, Ames, Iowa.

All remittances should be addressed to P. E. Brown, Bus. Mgr., Room 25 Hall of Agriculture, Iowa State College, Ames, Iowa.

Single Copies: One Dollar; Annual Subscription Three Dollars; In Canada, Three Dollars and a Quarter; Foreign, Three Dollars and a Half.

Entered as second class matter at the Post Office, Ames, Iowa.

## TABLE OF CONTENTS

### No. 1. October, 1931

The Action of a Transverse Electrostatic Field Upon Flames. J. K. McNEELY AND E. W. SCHILLING .....	1
The Introduction of Furyl-Alkyl Groups by Means of Sulfonic Esters. HENRY GILMAN AND ROBERT E. BROWN .....	11
The Propionic Acid Bacteria. I. Classification and Nomenclature. C. H. WERKMAN AND SARA E. KENDALL .....	17
The Literature of Alkylated Carbohydrates. V. Mono- and Di-Alkylated Glucose Derivatives. HAROLD W. COLES .....	33
The Literature of Alkylated Carbohydrates. VI. Tri-, Tetra-, and Penta-Alkylated Glucose Derivatives. HAROLD W. COLES.....	43
Studies on Sulfur Oxidation. DIONISIO I. AQUINO .....	65
Observations on <i>Bacillus calidolactis</i> . R. V. HUSSONG AND B. W. HAMMER .....	89
The Reaction of Cucumbers to Types of Mosaic. R. H. PORTER .....	95
<i>Eimeria separata</i> , a New Species of Coccidium from the Norway Rat ( <i>Epmys norvegicus</i> ). ELERY R. BECKER AND PHOEBE R. HALL....	131

### No. 2. January, 1932

Hydrogenation of Furfural. F. E. BROWN, HENRY GILMAN AND RALPH L. VAN PEURSEM .....	133
The Physiological Properties of Some Furan Derivatives. HENRY GILMAN, A. P. HEWLETT AND J. B. DICKEY.....	137
Certain Chemical and Morphologic Phases of the Blood of Normal and Cholera-infected Swine. I. The Concentration of Certain Chemical Constituents. E. A. HEWITT.....	143
Certain Chemical and Morphologic Phases of the Blood of Normal and Cholera-infected Swine. II. Certain Morphologic Phases. W. T. OGLESBY, E. A. HEWITT AND H. D. BERGMAN.....	227



### No. 3. April, 1932

Preliminary Experiments With Aphids as Vectors of Yellow Dwarf. C. J. DRAKE, H. D. TATE AND H. M. HARRIS.....	347
The Accuracy of the Dilution Method of Estimating the Density of a Population of Micro-organisms. EDWARD S. ALLEN.....	251
New and Rare North American Collembola. HARLOW B. MILLS.....	263
Nectaries of Capsicum. J. N. MARTIN, A. T. ERWIN AND C. C. LOUNSBERRY .....	277
The Relative Toxicity of Pyridine and Nicotine in the Gaseous Condition to <i>Tribolium confusum</i> Duval. CHARLES H. RICHARDSON AND LOUISE HAAS .....	287
Quantitative, Biometric and Host-Parasite Studies on <i>Eimeria miyairii</i> and <i>Eimeria separata</i> in Rats. E. R. BECKER, PHOEBE HALL AND ANNA HAGER .....	299
A Discussion of Synonymy in the Nomenclature of Certain Insect Flagellates, With the Description of a New Flagellate from the Larvae of <i>Ligyrodes relictus</i> Say (Coleoptera-Scarabaeidae). BERNARD V. TRAVIS .....	317
On the Coagulation of Blood from the Cockroach, <i>Periplaneta orientalis</i> (Linn.), With Special Reference to Blood Smears. J. FRANKLIN YEAGER, W. EARL SHULL AND MILTON D. FARRAR.....	325

### No. 4. July, 1932

First Supplementary List of Parasitic Fungi from Iowa. JOSEPH C. GILMAN.....	357
A Study of Caramel Color. JOHN B. SHUMAKER WITH J. H. BUCHANAN.....	367
Conjugated Systems in Furan Types. HENRY GILMAN AND JOSEPH B. DICKEY.....	381
The Stabilizing Effect of Nuclear Nitro Groups in Furan Types. 5-Nitro-2-Furfuryl Chloride and 5-Nitro-2-Furfuryl Methyl Ether. HENRY GILMAN AND ROBERT R. BURTNER.....	389
The Sulfur and Nitrogen of Wool. ELEANOR WINTON AND RACHEL EDGAR .....	395
Abstracts of Doctoral Theses .....	407



# THE ACTION OF A TRANSVERSE ELECTROSTATIC FIELD UPON FLAMES

J. K. MCNEELY<sup>1</sup> AND E. W. SCHILLING<sup>2</sup>

*From the Electrical Engineering Laboratory, Iowa State College*

Accepted for publication June 12, 1931

In an earlier paper, the action of a transverse electrostatic field upon a propane field was reported<sup>3</sup>. The results obtained with the propane flame seemed to indicate that the flame behavior was, to a large extent, dependent upon its carbon content. This paper reports the results obtained in using flames of varying carbon content. The flames were produced by burning the following: hydrogen, ethylene, methyl alcohol, methane, gasoline, acetylene, benzene, hydrogen sulphide and turpentine.

## THE COMBUSTION OF HYDROCARBONS

The phenomenon of combustion is not completely understood. However, great progress in establishing the underlying principles has been made as the result of studies extending over the last two and one-half centuries.

The separation of finely divided carbon in the thermal decompositions of the hydrocarbons is in all probability the cause of the luminosity of their flames. It seems established also that in general the hydrocarbons are more combustible than hydrogen in flames. This means that the hydrogen and oxygen do not combine to form steam as a final product when there is a deficiency of oxygen. The chemical reactions are complicated, i. e., many intermediate combinations take place. It is nevertheless interesting to note that for propane ( $C_3H_8$ ) an atom of oxygen may serve to break up the molecule forming a molecule of carbon monoxide and releasing two atoms of carbon which may account for the luminous carbon which does exist. On the other hand, the chemical reaction resulting from the union of oxygen with one molecule of propane may produce sufficient heat to decompose adjacent molecules by thermal decomposition. This thermal decomposition would account for the hydrogen and incandescent carbon.

In the above discussion, propane has been used as an illustration. Similar properties exist with the other hydrocarbons. The molecules of some gases are more stable than others. The possible products of chemical reactions differ with the various hydrocarbons. The stability of these products vary. Hence, some variation in the behavior of the hydrocarbon flames should be expected. Experiments show that such variations exist.

## APPARATUS AND PROCEDURE

The electrical equipment used in the present experiments was described in the companion paper to which reference has been made. A modification

<sup>1</sup>Professor of Electrical Engineering, Iowa State College.

<sup>2</sup>Instructor in Electrical Engineering, Iowa State College.

<sup>3</sup>The Action of a Transverse Electrostatic Field Upon a Propane Flame, by McNeely, Brown and Crosno, Iowa State College Jour. Sci., 5:269-283 (1931).

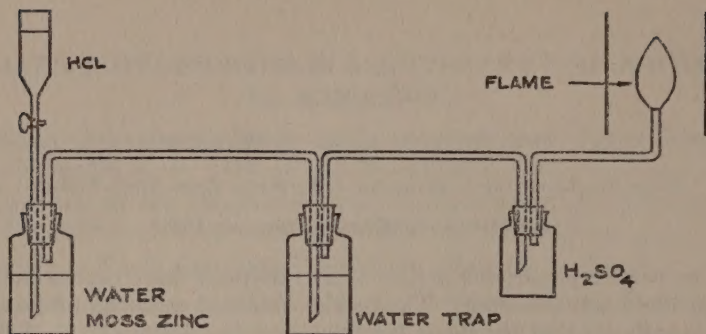


Fig. 1. Arrangement of Apparatus for Producing Hydrogen.

of standard methods was necessary for producing the gases used in the experiments reported in this paper. The combined use of flames and high voltage called for somewhat elaborate arrangements in order to insure safety. Figures 1, 2, 3, 6 and 10 are given to show how the gases were produced in successfully meeting the severe conditions imposed.

#### DESCRIPTION OF EXPERIMENTS

*Hydrogen.* Hydrogen gas was produced by pouring hydrochloric acid on moss zinc, using the apparatus shown in figure 1. A separatory funnel was filled with hydrochloric acid and the acid was allowed to run into the bottle containing the moss zinc and water in the necessary amounts to produce the required pressure in the gas. A water trap was used to prevent back firing of the gas when lighted. A small bottle containing the sulphuric acid to dry the gas was placed next to the flame. This also acted as a trap. A nickel blow pipe was used as a burner in order to obtain a pure hydrogen flame. When a glass tube is used sodium is given off, coloring the flame yellow. It was first thought that the sodium ions might cause the flame to be deflected. It was found, however, that the use of a glass tube did not

alter the results. The final test using hydrogen was made using a three-sixteenths inch copper tube.

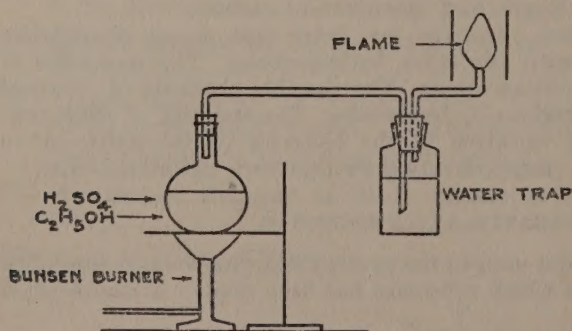


Fig. 2. Arrangement of Apparatus for Producing Hydrogen Ethylene Gas. By substituting  $\text{Na}_2\text{H}_3\text{O}_2$  and  $\text{NaOH}$  for  $\text{H}_2\text{SO}_4$  and  $\text{C}_2\text{H}_5\text{OH}$  this apparatus produced Methane Gas.

A hydrogen flame about two inches in height and about three-fourths inch in diameter showed no visible attraction toward either plate when placed midway between the plates. When placed about half an inch from the positive plate, the



flame behaved exactly as it did when no field was present. Likewise the field had no effect when the flame was placed one-half inch from the negative plate.

**Ethylene,  $C_2H_4$ .** This gas was produced by heating ethyl alcohol,  $C_2H_5OH$ , with sulphuric acid. The alcohol and by-products were removed by bubbling through water. The apparatus used was as shown in figure 2. In order to obtain a pressure steady enough to maintain a flame, the water bottle must not contain too much water, thus furnishing a storage space.

This gas was made in rather small quantities and hence the flame was smaller than used in the other experiments. It was about three-fourths of an inch high. It was attracted by the negative plate when placed in the center. It showed no attraction toward the positive plate.

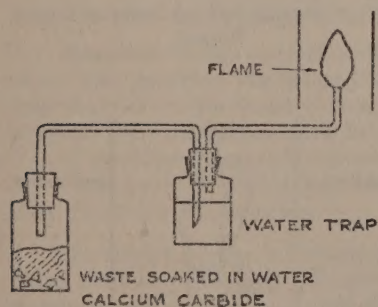


Fig. 6. Arrangement of Apparatus for Producing Acetylene Gas.

placed in a test tube and saturated with alcohol.

Using a flame about six inches high, it was noted that a high voltage produced a noticeable deflection which was toward the negative plate.

**Methane,  $CH_4$ .** This gas was made by heating sodium acetate and sodium hydroxide together and collecting the gas above water. It was found that this mixture must be heated for some little time before gas would be produced in sufficient quantity to maintain a flame.

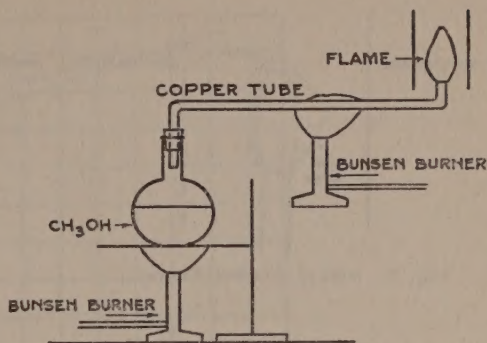


Fig. 3. Arrangement of Apparatus for Vaporizing Alcohol. Gasoline was vaporized with this apparatus by substituting the liquid gasoline for  $CH_3OH$ . Liquid Benzene was also vaporized using this apparatus.

**Methyl alcohol,  $CH_3OH$ .** A pure alcohol flame was obtained by heating alcohol in a Florence flask. It was found necessary also to heat the tube conducting the vapor from the flask to the flame in order to prevent condensation. To obviate this difficulty, a copper tube was used and this was heated. See figure 3. Wood alcohol was also burned from a paper wick. In this instance paper was

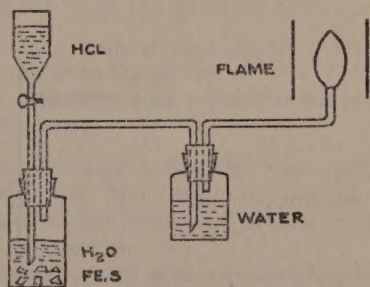


Fig. 10. Arrangement of Apparatus for Producing Hydrogen Sulphide.



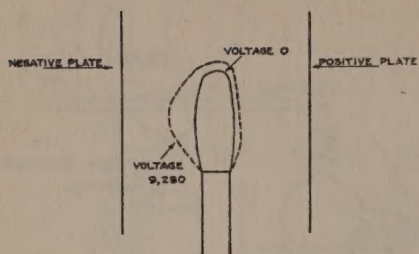


Fig. 4. Methyl Alcohol Flame.

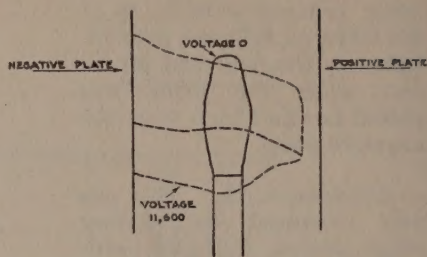


Fig. 5. Gasoline Flame.

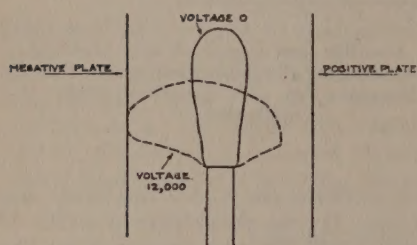


Fig. 7. Acetylene Flame.

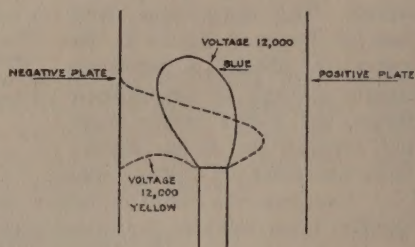


Fig. 8. Propane Flame from a Bunsen Burner.

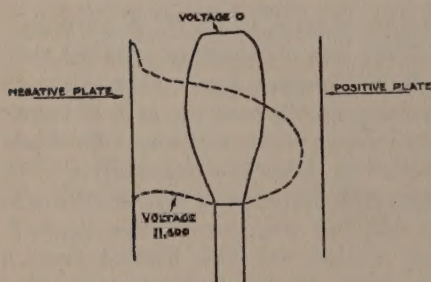


Fig. 9. Benzene Flame.

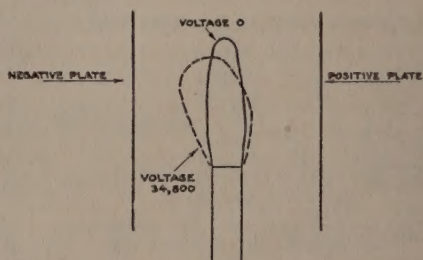


Fig. 11. Hydrogen Sulphide Flame.

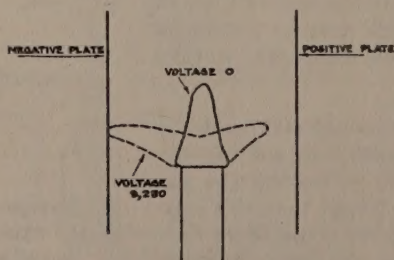


Fig. 12. Turpentine Flame.

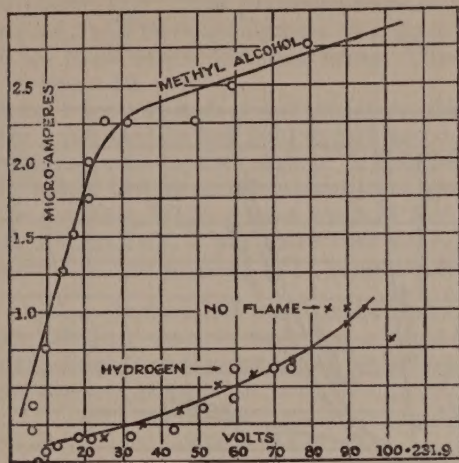


Fig. 13. Ionization Produced by Hydrogen and Methyl Alcohol Flames.

The yield of methane gas was small, but it was apparent that the flame was attracted to the negative plate.

*Gasoline*,  $C_6H_{14} - C_7H_{16}$ . This flame was produced by vaporizing gasoline. The gasoline was placed in a Florence flask and heated. The vapor was carried to the flame through a copper tube which could be heated to prevent the flame from back firing.

A gasoline flame placed midway between the plates was found to be attracted by both plates, but showed a greater attraction for the negative than for the positive plate.

*Acetylene*,  $C_2H_2$ . Production of this gas was obtained by dropping calcium carbide into water. However, it was found that this gave a varying pressure, which was not desirable. A piece of waste was moistened with water and placed in the bottle with a small piece of calcium carbide. A trap was used. Figure 6 shows this arrangement, which gave a very satisfactory flame.

This flame duplicated the results obtained with the gasoline flame.

*Propane*,  $C_3H_8$ . This was obtained from a tank containing the liquified gas and was fed through low pressure valves to the flame.

Figure 8 shows the action of the flame burning from a Bunsen burner both with the lower orifice open and with it closed.

*Benzene*,  $C_6H_6$ . A benzene flame was produced using the vaporized liquid. The apparatus used is as shown in figure 3. Here also a copper tube was used to conduct the vapor from the flask to the flame and means were provided for heating the tube to prevent condensation.

A flame obtained by vaporizing this liquid burned with an exceedingly sooty flame. In general, the results were the same as obtained with gasoline, acetylene and propane, except that this flame was attracted even more than the others.



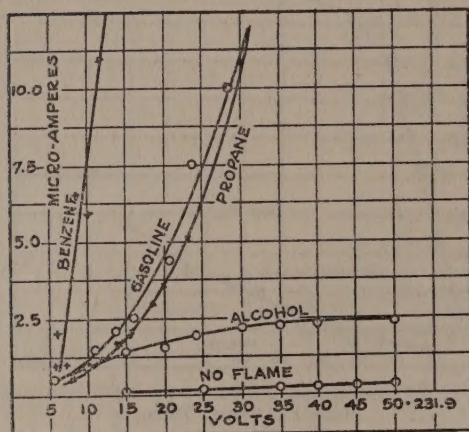


Fig. 14. Ionization Produced by Different Flames.

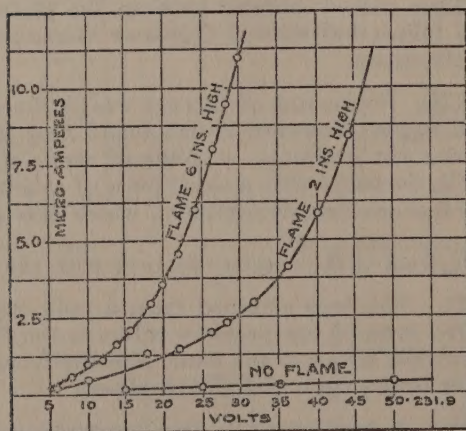


Fig. 15. Ionization Produced by Flames of Different Heights.



*Hydrogen Sulphide,  $H_2S$ .* This gas was produced from a mixture of dilute hydrochloric acid and ferrous sulphide. A trap was inserted between the generator and the flame to prevent back firing. This apparatus was arranged as shown in figure 10.

This gas was used because it does not contain carbon. Pure hydrogen gas was apparently unaffected by the field and it was desired to determine whether or not sulphur would act like carbon. It was found that this flame when placed in the center was attracted toward the negative plate and very slightly toward the positive. When placed one-half inch from the negative and positive plates, respectively, it was noted that although it was doubtless affected to some extent, it was so small as to be undetected.

*Turpentine,  $C_{10}H_{14}$ .* Turpentine was burned from a wick. Boiling it would not produce sufficient vapor to maintain a flame.

The flame was smokier than that produced by benzene. It was attracted in exactly the same manner as gasoline, acetylene, propane and benzene.

#### SUMMARY

The hydrogen flame was not affected by the electrostatic field. The hydrogen molecule cannot take on an extra electron and remains neutral.

In other words, no ionization takes place. This is shown by the amount of current required to maintain the voltage on the plates. It was found to be the same with the flame interposed as without the flame.

This experiment also shows that there is no such thing as an "electric wind" in the sense indicated by some writers. Foster and Porter have written in their textbook, "Electricity and Magnetism," ". . . by connecting a point and a plate with two sides of an electric machine, the electric field between them is constantly reproduced, and discharge takes place continuously. The air close to the point having given way, the stress in the air farther off has nothing to counterbalance it, and the air consequently moves in a continuous stream away from the point, constituting what is known as the electric wind." The fact that the hydrogen flame is not deflected, shows that there is no transverse current of air. When a hydrocarbon flame is used, there is a current of air in the direction of the deflection, but this is due to the motion of the burning gas. The current of air does not produce the deflection, but results from it.

A galvanometer in the line from the negative plate to ground gave the values of the current for the various experiments. If the amount of free carbon in a flame may be judged by the color of the flame, the ionization as measured by the current bears a direct relation to the free carbon. Figure 15 shows that a small flame produces a large amount of ionization. Also, the amount of ionization is decidedly greater for the larger flames.

## BIBLIOGRAPHY

## A. The Electrostatic Field and Electron Theory

1. BERTHOUD, A.  
1924. New theories of matter and the atom. George Allen and Unwid Ltd., London, p. 259.
2. FOSTER, G. C., AND A. W. PORTER  
1903. Electricity and magnetism. Longmans Green and Co., London, p. 568.
3. LAWS, F. A.  
1917. Electrical measurements. McGraw Hill Book Company, New York, p. 706.
4. LOEB, L. B.  
1927. Kinetic theory of gases. McGraw-Hill Book Company, New York, p. 555.
5. LORING, F. H.  
1921. Atomic theories. E. P. Dutton and Co., New York, p. 218.
6. MILLIKAN, R. A.  
1917. The electron. University of Chicago Press, Chicago, p. 266.
7. PEEK, F. W.  
1929. Dielectric phenomena in high voltage engineering. McGraw-Hill Book Co., New York, p. 265.
8. THOMPSON, J. J.  
1893. Recent researches in electricity and magnetism. Oxford at the Clarendon Press, p. 578.
9. THOMSON, J. J.  
1923. The electron in chemistry. The Franklin Institute, Philadelphia, p. 144.
10. TOWNSEND, J. S.  
1910. Theory of ionization of gases by collision. Constable and Co., London, p. 88.
11. WARNER, E. H., AND J. KUNZ  
1919. Corona discharge. Ill. Eng. Exp. Sta., Urbana, Bull. 114:138.
12. WHITEHEAD, S.  
1927. Dielectric phenomena and electrical discharges in gases. Ernest Benn Ltd., London, p. 175.
13. WHITEHEAD, J. B.  
1927. Lectures on dielectric theory and insulation. McGraw-Hill Book Co., New York, p. 154.

## B. Electrical Precipitation of Suspended Matter

1. BERG, G.  
1928. Precipitation in Europe. Elec. World, 92:499-503.
2. BUSH, H. J.  
1920. Electrostatic separation. Electrician, 85:533-535.
3. GELLERT, N. H.  
1924. Removing dust from blast furnace gases. Iron Age, 113:422-425.
4. HANLEY, H. R.  
1926. Electrical precipitation as applied to gas streams. Missouri Univ. School of Mines and Metallurgy, Tech. Ser., 9:1-64.

---

Note: Only those references not listed in the paper, "The Action of a Transverse Electrostatic Field Upon a Propane Flame," by McNeely, Brown and Crosno, are included here.

5. HARWOOD, P. B.  
1926. Automatic control for precipitation; cleaning of furnace gases by Cottrell method. *Iron Age*, **118**:1345-1346.
  6. PIER, H. M.  
1927. Electrical precipitation in the manufactured gas industry. *Gas Age*, **59**:705-707.
  7. SCHMIDT, W. A.  
1924. Electrical precipitation in retrospect. *Ind. and Eng. Chem.*, **16**:1038-1041.
  8. SULTZER, N. W.  
1928. Cottrell electrical precipitation processes. *Gas Age*, **61**:505-506.
  9. TOLMAN, R. C., AND S. KARRER  
1920. Motion of droplets and particles in the field of corona discharge. *Chem. and Met. Eng.*, **22**:1203-1206.
  10. WINNIE, H. A.  
1921. Cottrell process of electrical precipitation. *Gen. Elec. Rev.*, **24**:910-921.
- C. Flames and Flame Behavior in Electrostatic Fields
1. SMITH, ALEX  
1906. *Inorganic chemistry*. The Century Book Co., New York, p. 780.





# THE INTRODUCTION OF FURYL-ALKYL GROUPS BY MEANS OF SULFONIC ESTERS

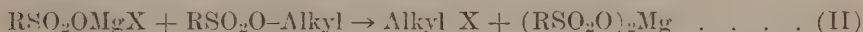
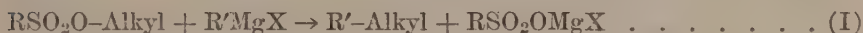
HENRY GILMAN AND ROBERT E. BROWN

*From the Chemical Laboratory of Iowa State College*

Accepted for publication August 3, 1931

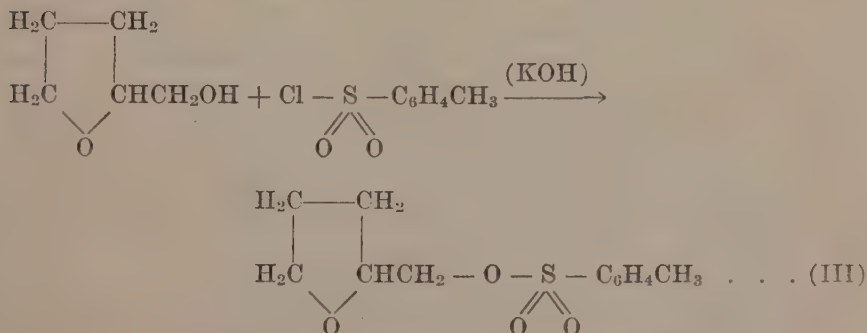
## INTRODUCTION

One of the outstandingly important reactions of organic chemistry is alkylation, and the widest applications of such reactions as Friedel-Crafts, Wurtz-Fittig, Grignard, acetoacetic and malonic ester condensations, etc., turn on such alkylation. In general, esters of inorganic acids like the halogen and sulfonic acids are used for this purpose. The high success attending alkylation by means of sulfonic esters and organomagnesium halides<sup>1</sup>,

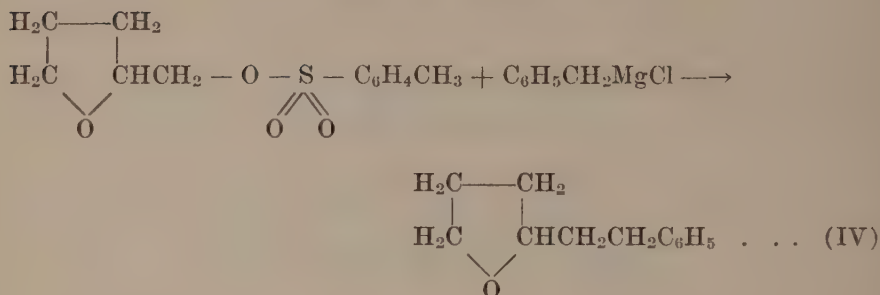


suggests its use with furan compounds.

The present study shows that this reaction does indeed occur with furan compounds, and that the method (and others using sulfonic esters) are of promise in extending the introduction of furyl-alkyl or tetrahydrofuryl-alkyl groups. Naturally, the first acid studied was the accessible *p*-toluenesulfonic acid or its acid chloride, *p*-CH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>SO<sub>2</sub>Cl. The following transformations have been effected.



<sup>1</sup>Gilman and Heck, *Ber.*, **62**, 1379 (1929) and *J. Am. Chem. Soc.*, **50**, 2223 (1928). These articles contain leading references to earlier work.



The tetrahydrofurfuryl *p*-toluenesulfonate (III) underwent considerable decomposition when attempts were made to distill it under reduced pressure. Fortunately, this was not a serious disadvantage because it was found that the oily ester could be conveniently purified by heating it in an oil bath at about 145-150°/1 mm. to remove volatile impurities. The yield was excellent (in excess of 90%). When treated with benzylmagnesium chloride, tetrahydrofurfuryl-phenyl-methane (IV) resulted.

Some orienting studies made by J. B. Dickey on the attempted synthesis of furfuryl *p*-toluenesulfonate indicate that the ester is probably first formed and then undergoes subsequent reaction with sodium furfurylate to give a di- $\alpha$ -furfuryl ether  $(\text{C}_4\text{H}_3\text{OCH}_2)_2\text{O}$ . One of the procedures involved the interaction of sodium furfurylate and *p*-toluenesulfonyl chloride in an inert medium. Under related conditions, Ferns and Lapworth<sup>2a</sup> obtained dibenzyl ether and postulated the intermediate formation of benzyl *p*-toluenesulfonate. Di- $\alpha$ -furfuryl ether was prepared earlier in another way by Zanetti<sup>2b</sup>.

Incidental to these studies, tetrahydrofurfuryl chloride, bromide and iodide were prepared<sup>3</sup>. These halides were treated, in some orienting experiments, with magnesium in ether with a view to preparing the corresponding organomagnesium halides. The encouraging progress with furfuryl chloride,  $\text{C}_4\text{H}_3\text{OCH}_2\text{Cl}$ , in related experiments<sup>4</sup> warrants the expectation that these  $\text{RMgX}$  compounds will form. When optimal conditions for their preparation have been developed the reagents should prove of considerable value in extending the utility of furan compounds.

Another important alkylation reaction is that of Friedel-Crafts. This reaction has been most extensively applied with aromatic compounds like benzene. In connection with a corresponding study with furan compounds (which have so much in common with benzene types), it is interesting to observe that Mr. N. O. Calloway has already shown that the alkyl group can be introduced in the furan nucleus (probably in the 5-position) when alkyl furoates are treated with alkyl halides and aluminum chloride. These reactions are being extended to include other furan types as well as other reactants like acid chlorides, acid anhydrides, etc.

<sup>2a</sup>Ferns and Lapworth, *J. Chem. Soc.*, **101**, 273 (1912). <sup>2b</sup>Zanetti, *J. Am. Chem. Soc.*, **49**, 1061, 1065 (1927). The authors are grateful to Dr. Zanetti for a sample of his di- $\alpha$ -furfuryl ether.

<sup>3</sup>Dox and Jones, *J. Am. Chem. Soc.*, **50**, 2033 (1928) prepared the bromide from the alcohol and hydrobromic acid or phosphorus tribromide. Kirner, *ibid.*, **52**, 3251 (1930) prepared the chloride from the alcohol and thionyl chloride (in good yields) and phosphorus trichloride.

<sup>4</sup>Gilman and Burtner, *Iowa State College Jour. Sci.*, **5**, 189 (1931).



## EXPERIMENTAL PART

*Tetrahydrofurfuryl p-Toluenesulfonate*,  $C_4H_7OCH_2OSO_2C_6H_4CH_3$ .—The general procedure was that used earlier<sup>5</sup> for the preparation of other esters.

To a solution of 95.3 g. (0.5 mole) of *p*-toluenesulfonyl chloride in 350 cc. of dry ether in a 500 cc. Erlenmeyer flask were added 51 g. (0.5 mole) of tetrahydrofurfuryl alcohol. The solution was cooled in an ice-salt bath to zero and finely powdered potassium hydroxide was added in small portions until 56 g. (1.0 mole) had been added. During the addition the temperature of the solution was not allowed to rise above 5°. The flask was well stoppered and it was allowed to stand, with frequent shaking, for two hours in an ice bath. After the reaction had ceased the contents of the flask were creamy-white and somewhat viscous. The mixture was poured into 800 cc. of ice water and it was then well stirred. The ether layer was separated and the water layer was extracted twice with 100 cc. and 50 cc. of ether, respectively. The combined ether extracts were dried over potassium carbonate and the ether was distilled off on the water bath. The remaining mass was a light yellow, somewhat viscous liquid. The last traces of ether were removed by heating the liquid on the water bath at the pressure of a good water pump. This liquid was divided into equal portions and the distillation of each portion was attempted.

The first portion of the ester was placed in a 500 cc. Claisen flask, which was heated by an oil bath, and distillation was attempted at 1 mm. pressure. The temperature of the oil bath was slowly raised and a small amount of distillate boiled over at 120°. While changing the receiver the ester in the Claisen flask decomposed violently. The distillation of the second portion of the ester was attempted under the same conditions except that no attempt was made to change the receiver. As before a small amount of distillate appeared at about 120° with the oil bath at about 145°. The temperature of the bath was slowly raised and at about 155° the ester in the flask commenced to darken very slightly. As the temperature of the bath was slowly increased the ester became darker and darker. At 180° it became so dark that the source of heat was removed. In spite of the removal of heat further decomposition took place and suddenly the ester foamed up into a dark brown, almost black, froth which quickly filled the whole distilling apparatus. The pressure on the distilling system, except at the very end, did not exceed 1 mm.

A second amount of the ester was made exactly according to the directions given above, but instead of trying to distill the product it was heated in an oil bath at 145-150°/1 mm. to remove volatile ingredients. The ester was cooled in the flask without releasing the pressure until all danger of decomposition had passed. The product was a light yellow, slightly viscous liquid. The yield of the ester was 119 g., 93 per cent of the theoretical.

*Anal.* Calcd. for  $C_{12}H_{16}O_4S$ . S, 12.50. Found: S, 12.09, 12.03.

*Reaction of Ester with Benzylmagnesium Chloride.*—To a solution of 0.5 mole of benzylmagnesium chloride in 400 cc. of ether, a solution of 102.4 g. (0.4 mole) of tetrahydrofurfuryl *p*-toluenesulfonate in an equal volume

<sup>5</sup>Gilman and Beaber, *J. Am. Chem. Soc.*, **47**, 518 (1925).

of ether was slowly added with stirring. A gentle reaction took place when the ester was added to the Grignard reagent and after about half of the ester had been added the mixture in the flask separated into two layers, the lower one a heavy, viscous paste, the upper a mobile ether solution. After the whole of the ester had been added the mixture was stirred as well as could be done for three hours. As much of the mixture as could be poured out of the flask was hydrolyzed by means of ice water; that remaining in the flask was hydrolyzed by adding ice to the flask. These combined portions were extracted well with ether and the ether solutions were dried over anhydrous potassium carbonate. After the ether had been distilled from the solution the remaining oil was distilled at 14 mm. At this pressure 15 g. of a pale yellow liquid distilled at 133°. This was tetrahydrofurfuryl-phenyl-methane. A large, almost black, tarry residue could not be distilled or hydrolyzed by sodium hydroxide. No halide was obtained.

*Anal.* Calcd. for  $C_{12}H_{16}O$ , C, 81.81; H, 9.16. Found: C, 81.69; H, 9.20.

*Preparation of Tetrahydrofurfuryl Halides,  $C_4H_7OCH_2X$ .*—The preparation of these halides was developed incidental to a series of studies on the splitting of the furan and tetrahydrofuran nuclei. None of the chloride was isolated when a benzene solution of the alcohol was treated with hydrogen chloride. The chloride was then made by the thionyl chloride-pyridine method and its properties and analysis agreed with those described by Kirner<sup>3</sup>.

The bromide was prepared incidental to the same studies from tetrahydrofurfuryl alcohol and hydrogen bromide<sup>3</sup>. One-half mole, 51 g., of tetrahydrofurfuryl alcohol in 50 cc. of benzene was cooled in ice and then saturated with dry hydrogen bromide. As in the other cases of the treatment of the alcohol with a hydrogen halide the solution soon turned dark. After standing for 24 hours at room temperature in a closed flask the solution was gently refluxed for three hours while a slow stream of hydrogen bromide was passed into the flask. The mixture was fractionally distilled at 12 mm. pressure. A considerable fraction was obtained boiling between 65° and 72°. This fraction contained much halogen and it was only partly miscible with water. Therefore it was well shaken with water and the insoluble layer was separated and dried with calcium chloride. On distillation at atmospheric pressure it boiled at 160-161°. This distillate was analyzed by the Carius method and it was found to be tetrahydrofurfuryl bromide<sup>3</sup>. The yield of bromide amounted to 17 g., or 20.6 per cent of the theoretical yield.

In an attempt to prepare tetrahydrofurfuryl iodide by the action of dry hydrogen iodide on the tetrahydrofurfuryl alcohol no iodide was obtained. The reaction produced a great amount of black decomposition products, and about 50 per cent of the alcohol was recovered. Therefore, the preparation of the iodide was attempted by a metathetical reaction of the chloride and sodium iodide. Twelve g. (0.1 mole) of tetrahydrofurfuryl chloride was added to a solution of 16 g. (0.2 mole) of dried sodium iodide in 80 cc. of pure acetone in a 150 cc. Erlenmeyer flask. The flask was tightly stoppered and put in a dark place. After several days a small, white precipitate had appeared and the solution was light brown in color. At the end of two weeks the solution had turned to a dark brown and the precipitate had increased considerably in amount. The acetone was then distilled off with a water pump and the residue was treated with 50 cc. of water to

dissolve the sodium halides. This mixture was thrice extracted, first with 30 cc. of ether, and twice with 10 cc. portions of ether each time. The combined ether extracts were dried by shaking with calcium chloride in a separatory funnel for about one-half hour. Distillation at 3 mm. pressure produced 6 g. of unchanged chloride, and 4 g. of an almost colorless liquid at 69-70°. The distillate darkened slowly on exposure to air. This 4 g. distillate was the tetrahydrofurfuryl iodide.

*Anal.* Calcd. for  $C_5H_9OI$ : I, 60.09. Found: I, 59.54.

#### SUMMARY

The alkylating action of sulfonic esters and organomagnesium halides has been shown to apply with tetrahydrofurfuryl *p*-toluenesulfonate. Furfuryl alcohol with *p*-toluenesulfonyl chloride probably first give the corresponding sulfonate which then undergoes subsequent reaction to yield difurfuryl ether. In attempts to split the ring with halogen acids, tetrahydrofurfuryl bromide was prepared from tetrahydrofurfuryl alcohol and hydrogen bromide. Tetrahydrofurfuryl iodide, prepared from tetrahydrofurfuryl chloride and sodium iodide, is less stable than the corresponding chloride and bromide.





# THE PROPIONIC ACID BACTERIA

## I. CLASSIFICATION AND NOMENCLATURE

C. H. WERKMAN AND SARA E. KENDALL

*From the Department of Bacteriology, Iowa State College*

Accepted for publication August 11, 1931

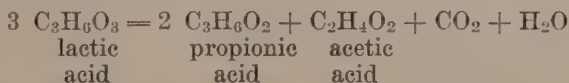
The first attempts to isolate and classify bacteria producing propionic acid as a principal product of their fermentation were carried out by Orla-Jensen working with von Freudenreich in 1906. These investigators divided their cultures of propionic acid bacteria into three groups based upon morphological differences:

(1) *Bacterium acidi propionici a*—a very short rod or stretched coccus, easily confused with *Streptococcus lactis* (*Bacterium lactis acidi*).

(2) *Bacterium acidi propionici b*—a short rod similar to *Bacterium acidi propionici a*, but when grown at a higher temperature (35-37°C.), exhibiting long, irregular rods or involution forms.

(3) *Bacillus acidi propionici*—a plump irregular or slightly curved rod, less anaerobic than the other two forms.

These investigators found all three forms to be easily stained, Gram positive, non-motile, gelatin non-liquefying, facultative anaerobes, having the ability to ferment lactates with the formation of propionic, acetic and carbonic acids. A quantitative determination showed that for each molecule of lactic acid which is split and oxidized to form acetic and carbonic acids, two other lactic acid molecules are reduced to propionic acid according to the following equation:



The three groups showed different reactions in milk: *Bacterium acidi propionici a* did not coagulate milk, *Bacterium acidi propionici b* did only after several days, and *Bacillus acidi propionici* did after two days.

Orla-Jensen (1909) grouped the propionic acid bacteria into a genus for which he proposed the name *Propionibacterium*. He believed that bacteria should be classified on the basis of their physiological characteristics, and stated that since the propionic acid bacteria distinguish themselves by oxidizing as well as reducing the cleavage products, as do the coli organisms, it is natural to separate them by these properties, therefore, the genus takes an intermediate place between the aerogenes and the lactic acid bacteria.

Thöni and Allemann (1908) were attracted by peculiar red and brown spots appearing on the cut surface of Emmental cheese. These spots proved to be almost pure cultures of organisms showing great similarity to von Freudenreich and Orla-Jensen's propionic acid bacteria. Thöni and Allemann's organisms produced a distinct red and brown pigment. They suggested the names *Bacterium acidi propionici* var. *rubrum* and *Bacterium*

*acidi propionici* var. *fuscum* for their organisms. Their descriptions do not permit adequate identification of these two organisms.

Troili-Petersson (1909) using a slightly alkaline medium, isolated, from Swedish cheeses, in addition to types 'a' and 'b' a new type which she called *Bact. acidi propionici c*, differing in its fermentative behavior.

Wolff (1912) isolated a few cultures of organisms capable of producing propionic, acetic and carbonic acids from lactates. Wojtkiewicz (1923) isolated and identified several cultures of *Bact. acidi propionici a* from Russian cheeses. Neither worker contributed to systematic knowledge of the propionic acid bacteria.

As a result of the work of Sherman and co-workers (1921-1923) an additional type, 'd', was added to the list; this type differed in its action in milk and glycerol. Sherman designated his organism *Bact. acidi propionici d*.

Van Niel (1928) working in Kluyver's laboratory in Delft, is an important contributor to our knowledge of the propionic acid bacteria. His contribution to the classification and nomenclature of these organisms resulted from a study of 30 cultures. Van Niel recognizes eight species, one of which, *Propionibacterium technicum*, is new.

Van Niel accepts the generic ranking of the propionic acid organisms with the name *Propionibacterium* as proposed by Orla-Jensen in 1909, but substituted species names in conformity to scientific rules of nomenclature. His species and nomenclature are as follows:

*Propionibacterium Freudenreichii* van Niel, for *Bacterium acidi propionici a*, von Freudenreich and Orla-Jensen.

*Propionibacterium Jensenii* van Niel, for *Bacterium acidi propionici b*, von Freudenreich and Orla-Jensen.

*Propionibacterium Peterssonii* van Niel, for *Bacterium acidi propionici c*, Troili-Petersson.

*Propionibacterium Shermanii* van Niel for *Bacterium acidi propionici d*, Sherman.

*Propionibacterium pentosaceum* van Niel, for *Bacillus acidi propionici* von Freudenreich and Orla-Jensen.

*Propionibacterium rubrum* van Niel, for *Bacterium acidi propionici*, var. *rubrum*, Thöni and Allemann.

*Propionibacterium Thöni* van Niel, for *Bacterium acidi propionici*, var. *fuscum*, Thöni and Allemann.

*Propionibacterium technicum*, van Niel, a previously undescribed strain with the power to ferment starch.

The present systematic study of the propionic acid bacteria is based upon 32 cultures. It has been the purpose to work out a convenient and rational grouping of the cultures based upon physiological characters. This choice is made for the convenience of identification of cultures, since morphological differentiation of the species based upon involuntary changes in acid, or alkaline medium under aerobic or anaerobic conditions does not lend itself to practical utilization. There is a high degree of correlation between the morphological and physiological criteria, and it is not implied that disagreement exists.

Appreciation is expressed for cultures received as follows: Nos. 1, 2, 3, 5, 7, 8, 9, 10, 11, 12, 13, 14 and 15 from Dr. J. M. Sherman of Cornell University, Ithaca, N. Y. Nos. 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 from



Dr. C. B. van Niel of Stanford University, Palo Alto, California. No. 6 from Dr. L. A. Burkey of the Dairy Division, U. S. D. A., Washington, D. C.

#### KEY TO THE SPECIES OF PROPIONIBACTERIUM

A dichotomous key to the species of *Propionibacterium* has been constructed, based on physiological differences. A key to the propionic bacteria based upon morphological characters proves inconvenient and is liable to lead even experienced investigators into error in the identification of species. The advantages of a dichotomous key are apparent.

In the use of physiological characters, care has been taken to use only those characters in the key which give sharp reactions. The propionics are characterized by their ability to vigorously attack certain carbohydrates and to mildly attack others as judged by the acidity developed. A number of carbohydrates have not been used in the key for this reason.

Bergey's (1930) key to the species of the genus contains several impracticable differentiations, such, for instance, as the extensive use of pigment formation as well as the use of ratios of propionic to acetic acid. His primary separation of the group on the basis of pigment and non-pigment formation is undesirable in view of the facts (1) that the production of the deeper colored pigments is uncertain and not sharply differentiated, and (2) that all the propionics studied by the authors may be considered to produce some pigment. The color ranges gradually from a cream to a brownish red. Any extensive use of pigment formation leads to confusion. We have used it to separate two species, one *P. rubrum* producing a brownish red, and *P. raffinosaceum*, producing a cream to buff pigment.

Pigment has been described in terms of the color charts in Mulliken, Identification of Pure Organic Compounds, Vol. 1. 1904. J. Wiley and Sons.

#### KEY TO THE SPECIES OF THE GENUS PROPIONIBACTERIUM

##### A. Attacking sucrose and maltose

##### B. Attacking the polysaccharides (starch, dextrin, glycogen)

—*Propionibacterium technicum*

##### BB. Not attacking polysaccharides

##### C. Attacking xylose and arabinose. Nitrates reduced.

—*Propionibacterium pentosaceum*

##### CC. Not attacking xylose and arabinose. Nitrates not reduced.

##### D. Attacking raffinose

##### E. Pigment yellow

—*Propionibacterium raffinosaceum*

##### EE. Pigment red-brown

—*Propionibacterium rubrum*

##### DD. Not attacking raffinose

##### E. Attacking mannitol. Not attacking sorbitol

##### F. Attacking amygdalin and salicin

—*Propionibacterium Peterssonii*



FF. Not attacking amygdalin or salicin  
—*Propionibacterium Jensenii*

EE. Not attacking mannitol. Attacking sorbitol  
—*Propionibacterium Thönii*

AA. Not attacking sucrose and maltose

B. Attacking lactose. Nitrates not reduced  
—*Propionibacterium Shermanii*

BB. Not attacking lactose. Nitrates reduced  
—*Propionibacterium Freudenreichii*

Bergey separates *P. Thönii* from *P. rubrum* by the fact that the former produces acids in the ratio of 5:1 and the latter in the ratio of 3:1. Mannitol, amygdalin and raffinose, among others, can be used to effect this separation.

Certain conditions, necessary in view of the properties of the propionic acid bacteria, must be imposed in the use of this key. A yeast medium furnishes much better growth than peptone media, but has the disadvantage that it may develop an acidity upon standing, which may give a red color with Andrade's indicator. We add approximately 50 grams of dried yeast to a liter of distilled water, boil to break down the cells, and allow the mixture to infuse in a refrigerator for at least one day. The supernatant liquid is removed and to it are added the other constituents excepting the carbon source, which is sterilized separately in a small amount of distilled water and added later, generally at the rate of 0.5 per cent.

The basal medium consists of the yeast infusion to which have been added two grams  $K_2HPO_4$  per liter and adjusted to pH 7.2. Brom-thymol blue or Andrade's indicator may be used. However, to avoid confusion, ten cubic centimeters of a four-day culture showing good growth should be titrated. A substantial production of acid requiring at least 1.5 cc. of 0.1 N alkali to neutralize should be present. This procedure is especially necessary in the use of the polysaccharides, which, due to certain causes, may show the presence of a small amount of acid. This is especially true in the case of *P. pentosaceum*. Reduction of the indicator must be guarded against; it is frequently necessary to add indicator solution to the culture.

#### THE GENUS PROPIONIBACTERIUM

The name *Propionibacterium* was proposed in 1909 by Orla-Jensen for the group of rod-shaped organisms fermenting lactose and lactates with the formation of considerable quantities of propionic acid. Orla-Jensen's statement is:

"Da die Propionsäurebakterien wie die Coli-Bakterien sich dadurch auszeichnen, dass sie die gebildeten Spaltungsprodukte sowohl oxydieren als auch reduzieren können, ist es natürlich, auch sie von den letzteren abzuleiten. In kultureller Beziehung nehmen sie eine Zwischenstellung zwischen den Aerogenes-Bakterien und den Milchsäurebakterien ein, indem sie in der Stichkultur sich auf der Oberfläche um so weniger ausbreiten je grösser ihre Fähigkeit zur Bildung von Propionsäure ist. Sie bilden unbe-

wegliche Stäbchen oder Diplokokken, die völlig an *Streptococcus lacticus* erinnern. Sie vergären sowohl Laktose als auch Laktate. Sie greifen das Kasein nicht an, sondern spalten die von den peptonisierenden Bakterien gebildeten Produkte weiter. Wegen ihrer reduzierenden Eigenschaften entwickeln sie Schwefelwasserstoff, wenn sie Pepton zur Verfügung haben. Wir wollen die Gattung *Propionibacterium* nennen."

Later Orla-Jensen (1921) proposed *Propionococcus* as the name of a genus for the coccus forms. In view of the known morphology of the propionics the significance of the latter name is lost. Orla-Jensen's statement is as follows:

"Whereas the shape of cells was formerly used as a family character, I have adopted it only as a generic one, and if we do not want to further confine its significance and only consider it as a specific character, we shall doubtless have to set up the genus *Propionococcus* besides the genus *Propionibacterium*."

Buchanan in 1921 states:

"The status of the genus is doubtful, as no species is described or referred to."

Since then a number of workers have described new species and *Propionibacterium* was accepted by Van Niel (1928) to designate the genus.

Bergey (1930) places the genus as the eleventh in the family *Bacteriaceae*. Tribe *Propionibacterieae* has the one genus *Propionibacterium* with the characters of the tribe.

There is good reason to afford generic ranking to the group of propionic acid bacteria. They show a natural uniformity in both cultural and physiological characters. They are non-motile short non-sporulating rods or "stretched" cocci with involutionary changes depending upon acidity or oxygen tension.

All are gram positive, retaining this character even after death of the culture due to age (two years) in a refrigerator. All show metachromatic staining with Albert's stain resembling, in this respect and in their involution forms, *Corynebacterium diphtheriae*.

Physiologically they are characterized by the production of propionic, acetic and carbonic acids from carbohydrates, organic acids, polyalcohols and glucosides. All are catalase positive and grow under anaerobic conditions.

Generic Diagnosis: *Propionibacterium*, Orla-Jensen, 1909.

*Short rods, non-motile, non-sporulating, gram-positive, assuming involution forms in acid media or under aerobic conditions. Anaerobes, catalase positive, failing to liquefy gelatine or to produce indol. Attack carbohydrates, polyalcohols, glucosides and hydroxy and keto acids with the production of relatively large quantities of propionic acid with lesser amounts of acetic acid and CO<sub>2</sub>. Require complex organic nitrogen compounds. Mesophilic.*

The type species is *Propionibacterium Freudenreichii*.

#### DESCRIPTION OF THE SPECIES

Nine species are recognized in the genus based on a study of 31 cultures. Table 1 gives the fermentations in carbohydrate media.

*Propionibacterium Freudenreichii*  
van Niel, 1928

*Synonyms:* *Bacterium acidi propionici* a, von Freudenreich and Orla-Jensen, 1906, *Bacterium acidi propionici* var. *fuscum* Thöni and Allemann, 1908.

*Cultures:* 1, 2, and 28.

*Cultural Characteristics:*

Morphology: Medium: yeast-lactate-phosphate liquid.

Temperature: 30°C.

Form: Very short rods, nearly spherical.

Arrangement: Single.

Size: 0.5 x 0.6 $\mu$ .

Non-motile.

Staining: Gram positive.

Metachromatic granules with Albert's stain.

Growth: Liquid medium: distinctly turbid, ropy sediment, grayish cream.

Agar slants: Granular streaks.

Aerobic: Slight growth, cream color.

37°C.: Good growth.

Acid media: Cells nearly spherical, partly in chains.

Pigment formation: Cream colored to yellow (Mulliken, OY tint 2 to YO tint 1).

Litmus milk: Slight decolorization of indicator.

Potato: No growth.

*Biochemical Characters:*

Catalase positive.

Nitrates reduced to nitrites.

Indol negative.

Acetyl-methyl-carbinol not produced from dextrose.

Gelatin liquefaction: None.

*Fermentation Reactions:*

Acid from glucose, mannose, levulose, galactose, arabinose (sl.), esculin, glycerol, adonitol, inositol, and erythritol.

No acid from sucrose, maltose, lactose, trehalose, raffinose, melezitose, dextrin, glycogen, inulin, starch, xylose, rhamnose, salicin, amygdalin, dulcitol, mannitol, sorbitol, perseitol, pectin, and xylan.

*Propionibacterium Freudenreichii* conforms to the generic diagnosis. In addition it exhibits a slight aerobic development and a good growth at 37°C. and reduces nitrates to nitrites. Acetyl-methyl-carbinol is not produced from glucose; milk is not coagulated. Acid is produced from the hexoses, arabinose, esculin, glycerol, adonitol, inositol, and erythritol. No acid is produced from the disaccharides, trisaccharides, and polysaccharides.



*Propionibacterium Jensenii*  
van Niel, 1928

*Synonyms:* *Bacterium acidi propionici b*, von Freudenreich and Orla-Jensen, 1906.

*Cultures:* 27 and 32.

*Cultural Characteristics:*

*Morphology:*

Temperature 30°C.

Form: Short rods.

Arrangement: Single.

Size: 0.7 x 1.0-1.3 $\mu$ .

Non-motile.

Staining: Gram positive.

Growth: No marked involutionary forms in acid media.

Pigment formation: Yellow to orange (Mulliken: yellow-orange, normal tone in young cultures to orange-shade in old cultures).

*Biochemical Characters:*

Catalase positive.

Nitrates not reduced to nitrites.

Gelatin liquefaction: None.

*Fermentation Reactions:*

Acid from dextrose, levulose, galactose, saccharose, adonitol, maltose, lactose, raffinose (occasionally), esculin, mannose, glycerol, erythritol, inositol, mannitol, trehalose. No acid from xylose, arabinose, dextrin, starch, glycogen, inulin, dulcitol, amygdalin, melizitose, perseitol, pectin, raffinose, rhamnose, salicin, sorbitol and melibiose.

*Propionibacterium Jensenii* conforms to the generic diagnosis, and attacks the monosaccharides, disaccharides, and glycerol. It produces an orange-yellow pigment.

*Propionibacterium Peterssonii*  
van Niel, 1928

*Synonyms:* *Bacterium acidi propionici c*, Troili-Petersson, 1909.

*Cultures:* 11, 12, 24 and 25.

*Cultural Characteristics:*

*Morphology:* Medium: yeast-lactate-phosphate liquid.

Temperature: 30°C.

Form: Short rods.

Arrangement: Single, pairs.

Size: 0.7 x 2-3 $\mu$ .

Non-motile.

Staining: Gram positive.

Metachromatic granules.

Growth: Liquid medium: slightly turbid, sediment in flaky mass.

Agar slants: Beaded.

Aerobic: Good growth.

37°C. 11: Slight growth.

12: No growth.

24: Slight growth.

25: Slight growth.

Pigment formation: Cream yellow (Mulliken: OY yellow, tint 2).

Litmus milk: Slight decolorization, acid, and very slight coagulation.

Potato: Slight growth.

#### *Biochemical Characters:*

Catalase positive.

Nitrates not reduced to nitrites.

Indol negative.

Acetyl-methyl-carbinol not produced from glucose.

Gelatin liquefaction: None.

#### *Fermentation Reactions:*

Acid from glucose, adonitol, arabitol, amygdalin, mannose, levulose, galactose, sucrose, erythritol, maltose, lactose, trehalose, melezitose (slight), salicin, esculin, glycerol, mannitol, adonitol, inositol. No fermentation from raffinose, dextrin, glycogen, inulin, starch, xylose, arabinose, sorbitol, rhamnose, dulcitol, perseitol, pectin, melibiose and xylan.

*Propionibacterium Peterssonii* conforms to the generic diagnosis. In addition it exhibits a good aerobic growth, coagulates milk very slowly, does not reduce nitrates or produce acetyl-methyl-carbinol from glucose. Of the carbohydrates, the hexoses and disaccharides are attacked; also salicin, esculin, glycerol and mannitol. Raffinose, the polysaccharides, pentoses, pectin and xylan are not attacked.

*Propionibacterium Shermanii* van Niel, 1928

*Synonyms:* *Bacterium acidi propionici* d, Sherman, 1921.

*Cultures:* 3, 4, 5, 6, 26 and 21.

#### *Cultural Characteristics:*

Morphology: Medium: yeast-lactate-phosphate liquid.

Temperature: 30°C.

Form: Short rods.

Arrangement: Single.

Size: 0.5 x 0.6 $\mu$ .

Non-motile.

Staining: Gram positive.  
Metachromatic granules.

Growth: Liquid medium: Moderately turbid, ropy sediment.  
Agar slants: Moderately granular.  
Aerobic: Slight growth or none at all.  
37°C.: No growth.

Pigment formation: Slight, yellowish (Mulliken: Y, tint 2).

Litmus milk: Complete decolorization, acid, coagulation.

#### *Biochemical Characters:*

Catalase positive.  
Nitrates not reduced to nitrites.  
Indol negative.  
Acetyl-methyl-carbinol not produced from glucose.  
Gelatin liquefaction: None.

#### *Fermentation Reactions:*

Acid from glucose, mannose, arabitol, levulose, galactose, lactose, arabinose (slight), esculin, glycerol, adonitol, salicin (slight), inositol and erythritol. No acids from sucrose, maltose, trehalose, raffinose, melezitose, dextrin, glycogen, inulin, starch, xylose, rhamnose, amygdalin, dulcitol, mannitol, sorbitol, perseitol and pectin.

*Propionibacterium Shermanii* conforms to the generic diagnosis. In addition it exhibits no growth at 37°C., or under aerobic conditions. Coagulates milk and reduces nitrates to nitrites. It does not produce acetyl-methyl-carbinol from glucose. Acid is produced from hexoses, lactose, glycerol, adonitol, inositol and erythritol. No reaction is shown with the disaccharides (excluding lactose), the trisaccharides, polysaccharides, pectin and xylan.

#### *Propionibacterium pentosaceum* van Niel, 1928

*Synonyms:* *Bacillus acidi propionici* von Freudenreich and Orla-Jensen, 1906.

*Cultures:* 13, 20 and 31.

#### *Cultural Characteristics:*

Morphology: Medium: Yeast-lactate-phosphate liquid.  
Temperature: 30°C.  
Forms: Short rods.  
Arrangement: Single, pairs, short chains.  
Size: 0.7 x 1.0 $\mu$ .  
Non-motile.

Staining: Gram positive.  
Metachromatic granules.



Growth: Liquid medium: Slight turbidity, heavy ropy sediment.

Agar slants: Grayish, slightly granular.

Aerobic: Good growth, involution forms marked.

37°C.: Good growth.

Acid media: Cells branched, crooked, swollen.

Pigment formation: Slight yellowish (Mulliken: OY, tint 2).

Litmus milk: Slight decolorization of indicator, and coagulation.

Potato: Good growth.

#### *Biochemical Characters:*

Catalase positive.

Nitrates reduced to nitrites (culture No. 13 shows a strong reduction to nitrite).

Indol negative.

Acetyl-methyl-carbinol not produced from glucose.

Gelatin liquefaction: None.

#### *Fermentation Reactions:*

Acid from arabitol, glucose, mannose, levulose, galactose, sucrose, maltose, lactose, melibiose, trehalose, raffinose, melezitose, xylose, arabinose, rhamnose, salicin, esculin, amygdalin, glycerol, mannitol, adonitol, inositol, sorbitol and trythritol. No acid from dextrin, glycogen, inulin, dulcitol, perseitol or pectin. A very slight attack of starch is manifest.

*Propionibacterium pentosaceum* conforms to the generic diagnosis. In addition it exhibits a good aerobic and 37°C. development and coagulates milk. It does not produce acetyl-methyl-carbinol from glucose. The hexoses, disaccharides, trisaccharides, polyalcohols (with the exception of dulcitol and perseitol), pentoses and glucosides are attacked. No reaction is shown with the polysaccharides, pectin and xylan. Occasionally a weak acid reaction is observed in starch.

#### *Propionibacterium Thönii* van Niel, 1928

*Synonyms:* *Bacterium acidi propionici* var. *rubrum* Thöni and Allemann, 1908.

*Cultures:* 8, 10, 22, 23.

#### *Cultural Characteristics:*

Morphology: Medium: Yeast-lactate-phosphate liquid.

Temperature: 30°C.

Form: Short rod.

Arrangement: Short chains.

Size: 1.0-1.5 $\mu$ .

Non-motile.

Staining: Gram positive.  
Metachromatic granules.

Growth: Liquid medium: moderately turbid, abundant ropy sediment.

Agar slants: Coarsely beaded.

Aerobic: Good growth.

37°C.: Moderate growth.

Pigment formation: Dark red-orange (Mulliken: YO, normal to shade).

Litmus milk: Slight decolorization, slight acid, coagulation.

Potato: Good growth.

#### *Biochemical Characters:*

Catalase positive.

Nitrates not reduced to nitrites.

Indol negative.

Acetyl-methyl-carbinol produced from glucose.

Gelatin liquefaction: None.

#### *Fermentation Reactions:*

Acid from glucose, arabitol, mannose, levulose, galactose, sucrose, maltose, lactose, trehalose, salicin, inositol (slight), esculin, glycerol, adonitol, sorbitol and erythritol. No acid from raffinose, melezitose, dextrin, glycogen, inulin, starch, xylose, arabinose, rhamnose, amygdalin, dulcitol, mannitol, perseitol, pectin or melibiose.

*Propionibacterium Thönii* conforms to the generic diagnosis. In addition it exhibits a good aerobic and 37°C. development, produces an abundant reddish-orange pigment, coagulates milk, and produces acetyl-methyl-carbinol from glucose. Nitrates are not reduced to nitrites. The hexoses, disaccharides, salicin, esculin, glycerol, adonitol, sorbitol and erythritol are attacked. No reaction is shown with the trisaccharides, polysaccharides, pentoses, pectin and xylan.

*Propionibacterium technicum* van Niel, 1928

*Synonyms:* None.

*Cultures:* 14, 15 and 17.

#### *Cultural Characteristics:*

Morphology: Medium: Yeast-lactate-phosphate liquid.

Temperature: 30°C.

Form: Short rods.

Arrangement: Pairs.

Size: 0.5 x 0.6 $\mu$ .

Non-motile.

Staining: Gram positive.

Metachromatic granules.

Growth: Liquid medium: moderate turbidity, sediment extremely flocculent.

Agar slants: No growth.

Aerobic: No growth.

37°C.: No growth.

Pigment formation: Creamy yellow (Mulliken: Y0 tint 1 to normal).

Litmus milk: Slight decolorization, slight acid and coagulation.

Potato: Moderate growth.

#### *Biochemical Characters:*

Catalase positive.

Nitrates not reduced to nitrites.

Indol negative.

Acetyl-methyl-carbinol not produced from glucose.

Gelatin liquefaction: None.

#### *Fermentation Reactions:*

Acid from adonitol, glucose, arabitol, mannose, levulose, galactose, sucrose, amygdalin, maltose, lactose, raffinose, dextrin, glycogen, erythritol, starch, arabinose, salicin, esculin, glycerol, mannitol and inositol. No acid from trehalose, melezitose, inulin, xylose, rhamnose, dulcitol, adonitol, sorbitol, perseitol, pectin.

*Propionibacterium technicum* conforms to the generic diagnosis. In addition it exhibits a creamy yellow pigment. It does not reduce nitrates to nitrites or produce acetyl-methyl-carbinol from glucose; it does not coagulate milk. The hexoses, disaccharides (with the exception of trehalose), raffinose, the polysaccharides (with the exception of inulin), the glucosides and glycerol are attacked. No reaction is shown with xylose, rhamnose, pectin and xylan.

#### *Propionibacterium rubrum* van Niel, 1928

*Synonyms:* *Bacterium acidi propionici* var. *rubrum* Thöni and Allemann, 1908.

*Culture:* 9, 16 and 19.

#### *Cultural Characteristics:*

Morphology: Medium: yeast-lactate-phosphate liquid.

Temperature: 30°C.

Form: Short rods.

Arrangement: Single, pairs.

Size: 0.8 x 1.2 $\mu$ .

Non-motile.

Staining: Gram positive.

Metachromatic granules.



Growth: Liquid medium: very slight turbidity, sediment finely flocculent.

Agar slants: Very slightly beaded.

Aerobic: Good growth.

37°C.: No growth (9); moderate growth (19).

Pigment formation: Abundant reddish orange (Mulliken: O shade 1 to normal tone).

Litmus milk: Slight decolorization, acid and slow coagulation.

Potato: Moderate Growth.

#### *Biochemical Characters:*

Catalase positive.

Nitrates not reduced to nitrites.

Indol negative.

Acetyl-methyl-carbinol not produced from glucose.

Gelatin liquefaction: None.

#### *Fermentation Reactions:*

Acid from glucose, mannose, arabinol, levulose, galactose, sucrose, maltose, lactose, trehalose, raffinose, melezitose, amygdalin, glycerol, mannitol, adonitol, esculin, salicin, sorbitol and erythritol. No acid from dextrin, glycogen, inulin, starch, xylose, arabinose, rhamnose, dulcitol, perseitol, pectin, melibiose or inositol.

*Propionibacterium rubrum* conforms to the generic diagnosis. In addition it exhibits a good growth under aerobic conditions, and produces an abundant reddish-orange pigment. It does not reduce nitrates to nitrites or produce acetyl-methyl-carbinol from glucose; it coagulates milk very slowly. The hexoses, disaccharides and trisaccharides, amygdalin (slight) and polyalcohols with the exception of dulcitol, sorbitol and perseitol, are attacked. No reaction is shown with the polysaccharides, pentoses, pectin, melibiose or xylan.

#### *Propionibacterium raffinosaceum* sp. nov.

*Synonyms:* *Propionibacterium Jensenii* var. *raffinosaceum*, van Niel, 1928.

*Bacterium acidi propionici* b, von Freudenreich and Orla-Jensen (in part), 1906.

*Cultures:* 7 and 18 (considered as variety of *P. Jensenii* by van Niel).

#### *Cultural Characteristics:*

Morphology: Medium: yeast-lactate-phosphate liquid.

Temperature: 30°C.

Form: Short rods.

Arrangement: Single, short chains.

Size: 0.7 x 1.0-1.3 $\mu$ .

Non-motile.

Staining: Gram positive.

Metachromatic granules.

(Growth: Liquid medium: only slightly turbid, sediment flaky.

Agar slants: Moderately granular.

Aerobic: Good growth.

37°C.: No growth.

Pigment formation: Yellow (Mulliken: orange-yellow, tint 1).

Litmus milk: Complete decolorization of indicator, slight acid reaction, coagulation.

Potato: Good growth.

#### *Biochemical Characters:*

Catalase positive.

Nitrates not reduced to nitrites.

Indol negative.

Acetyl-methyl-carbinol not produced from glucose.

Gelatin liquefaction: None.

#### *Fermentation Reactions:*

Acid from glucose, arabitol, mannose, levulose, galactose, sucrose, maltose, lactose, trehalose, raffinose, melezitose, salicin, esculin, glycerol, mannitol, adonitol, inositol, erythritol, amygdalin. No action from dextrin, glycogen, inulin, starch, xylose, arabinose, rhamnose, dulcitol, perseitol, pectin, melibiose or sorbitol.

*Propionibacterium raffinosaceum* conforms to the generic diagnosis. In addition it exhibits a good growth under aerobic conditions, produces a yellowish pigment, and coagulates milk. It does not reduce nitrates to nitrites or produce acetyl-methyl-carbinol from glucose. The hexoses, disaccharides and trisaccharides, salicin, esculin, and the polyalcohols, with the exception of dulcitol and perseitol, are attacked. No reaction is shown with the polysaccharides, pentoses, pectin or xylan.

#### SUMMARY

The classification and nomenclature of the propionic acid-producing bacteria are discussed, based on a careful cultural study of 32 cultures.

The name *Propionibacterium* Orla-Jensen, 1909, is valid for the genus.

Nine species in the genus *Propionibacterium* are recognized and a key has been prepared based on physiological differences. One new species, *P. raffinosaceum*, is described.

#### REFERENCES

BERGEY, D. H.

1930. Manual of determinative bacteriology, 3 ed. 589 pp. Williams and Wilkins, Baltimore, Md.

BUCHANAN, R. E.

1921. General systematic bacteriology. 597 pp. Williams and Wilkins, Baltimore, Md.

FREUDENREICH, E. VON, UND S. ORLA-JENSEN

1906. Ueber die im Emmentalerkäse stattfindende Propionsäuregärung. Centralbl. f. Bakt. II, 17:529-546.

ORLA-JENSEN, S.

1909. Die Hauptlinien des natürlichen Bakteriensystems. Centralbl. f. Bakt. II, 22:305-346.

---

1921. The main lines of the natural bacterial system. J. Bact. 6:263-273.

SHAW, R. H., AND J. M. SHERMAN

1923. The production of volatile fatty acids and carbon dioxide by propionic acid bacteria, with special references to their action in cheese. J. Dairy Sc. 6:303-309.

SHERMAN, J. M.

1921. The cause of eyes and characteristic flavor in Emmental or Swiss cheese. J. Bact. 6:379-394.

---

AND R. H. SHAW

1921. Associative bacterial action in the propionic acid fermentation. J. Gen. Physiol. 3:657-658.

---

AND

1923. The propionic acid fermentation of lactose. J. Biolog. Chem. 56:695-700.

THÖNI, J., AND O. ALLEMAN

1908. Ueber rote Punkte in Emmentalerkäsen hervorgerufen durch *Bacterium acidipropionici* var. *rubrum*. Landw. Jahrebericht d. Schweiz. 22:45-51.

---

AND

1910. Über das Vorkommen von gefärbten makroskopischen Bakterienkolonien in Emmentalerkäsen. Centralbl. f. Bakt. II, 25:8-30.

TROILI-PETERSSON, G.

1909. Studien über in Käse gefundene glycerinvergärende und lactatvergärende Bakterien. Centralbl. f. Bakt. II, 24:333-342.

---

1915. Einzellkultur von langsam wachsenden Bakterienarten, speziell der Propionsäurebakterien. Centralbl. f. Bakt. II, 42:526-528.

VAN NIEL, C. B.

1928. The propionic acid bacteria, N. V. Uitgeverszaak, J. W. Boissevain and Co. Haarlem.

WOJTKIEWICZ, A.

1923. Propionsäuregärung im Emmentaler Käse russischer Fabrikation. Centralbl. f. Bakt. II, 59:333-336.

WOLFF, O.

1912. Sauerungsbakterien, insonderheit Milchsäurelangstäbchen und Propionsäurebildner in Molkereiprodukten, speziell in den verschiedenen Käsesorten. Centralbl. f. Bakt. II, 34:494-540.



# THE LITERATURE OF ALKYLATED CARBOHYDRATES

## V. MONO- AND DI-ALKYLATED GLUCOSE DERIVATIVES

HAROLD W. COLES

*From the Laboratory of Plant Chemistry, Department of Chemistry, Iowa State College*

Accepted for publication August 1, 1931

### INTRODUCTION

The literature on alkylated glucoses is more extensive than that of any other sugar due to the more common occurrence of glucose, either alone or combined with other sugars. This has resulted in closer agreement among the workers with regard to the structure of the methylated sugars. The original papers should be consulted with regard to these structural arguments.

### MONO-ALKYL GLUCOSSES

A monomethyl methyl glucoside has been reported (47) among the products of methylated glycogen. Hydrolyzed methylated tetra-amylose (89) yields 7 per cent of monomethyl glucose. An uncrystallizable syrup, a monomethyl glucose, which reduces Fehling's but does not yield an osazone, is secured from glucosan (16, 20). An amorphous monomethyl glucose is mentioned (15, 73). In the hydrolysis of incompletely methylated cellulose, an amorphous monomethyl glucose or mixture of monomethyl glucoses was isolated. An osazone was prepared (7, 18). Completely methylated cellulose is stated to not give a monomethylated glucose (36).

The following steps have been carried out, but the structures have not been elaborated (21, 43):—Anhydro-methyl glucoside mono-oleate  $\rightarrow$  *monomethyl anhydromethyl glucoside mono-oleate*  $\rightarrow$  *monomethyl anhydromethyl glucoside*  $\rightarrow$  *monomethyl methyl glucoside*  $\rightarrow$  *methyl glucose*. A *monoacetyl, tribenzoyl monomethyl glucose* is mentioned (90), and also an *ethyl aminoglucose* (5). Monomethyl glucoses are mentioned but not specifically described in 27, 31, 66 and 78.

#### *2-Methyl Glucose*

This sugar (50) has been obtained from the following sources:

- (a)—Monomethyl dicellulose (92).
- (b)—3,4,6-Triacetyl 2-methyl methyl glucoside (79, 85, 92).
- (c)—2-Methyl-glucose diethyl mercaptal (96, 99, 100, 104, 105).
- (d)—An alcoholic solution of glucosan and sodium (13, 14, 16, 20 and 96).

It is a colorless syrup (13, 14, 92), amorphous (79, 85, 95), with a melting point of 158° (96, 105). It is soluble in water, less so in alcohol and acetone, and only slightly soluble in ethyl acetate even when boiling (96). The rotation was found to be  $[\alpha]_D^{20} + 59.2^\circ \rightarrow + 64.9^\circ$  (water) (96) and  $[\alpha]_D^{23} + 21.1^\circ$  (water)  $\rightarrow + 62.1^\circ$  (105). It does not react with fuchsin

and sulphuric acid (96). Various modifications are said to exist (79, 96). Although no *osazone* is possible (13, 14, 16, 20, 92 and 95), a readily crystallizable *phenylhydrazone* may be secured (104). This has a melting point of  $177^{\circ}$  (96, 99, 100, 105);  $175.6^{\circ}$  (79, 92);  $178^{\circ}$  (85); consists of needles difficultly soluble in water and ether, and somewhat soluble in alcohol. The rotation in the last solvent named is  $-12.3^{\circ}$  at  $17^{\circ}$  (85). It exhibits a rotation of  $-9^{\circ}$  in pyridine (105).

A methyl hexosazone was reported as being formed by 2-methyl glucose by Paesu (55) and because of this he stated it to be 4-methyl glucose. Later workers (104, 105) could not verify this, but obtained an osazone identical with glucosazone. On oxidation with nitric acid, 2-methyl glucose gives *methyl saccharic acid* isolated as the *Ca salt*.

The following derivatives have been described :

(a)—*2-Methyl-3, 4, 5, 6-tetrabenzoyl glucose ethyl mercaptal* (96).

This consists of large, glittering prisms, melting point  $88-89^{\circ}$ , easily soluble in chloroform, acetone, ethyl acetate, hot methyl and ethyl alcohols, and difficultly soluble in ligroin.

(b)—*2-Methyl glucose diethyl mercaptal* (96, 99, 100).

Melting point  $178^{\circ}$ . Difficulty soluble in most solvents, easily in warm water and alcohol.

(c)—*3, 4, 6-Triacetyl, 2-methyl methyl glucoside* (79, 85, 92).

Said to be small needles, showing a melting point of  $74-75^{\circ}$ ;  $95-96^{\circ}$  (79);  $121^{\circ}$  (uncorr.) (92); and easily soluble in benzene, acetone, ether, alcohol, carbon tetrachloride, less soluble in chloroform, and difficultly soluble in water and ligroin. Doesn't reduce Fehling's, and exhibits no mutarotation (85). Rotation is  $+5.0^{\circ}$  (alcohol) (79);  $+5.88^{\circ}$  (alcohol) (85), and  $+6.28^{\circ}$  (alcohol) (85).

(d)—*2-Methyl methyl glucoside* (85).

This sugar does not reduce Fehling's as would be expected, and crystallizes with one-half mol of water,  $[\alpha]_D^{17} -23.9^{\circ}$ . The melting point is  $95-97^{\circ}$ .

(e)—*A diacetone derivative* is mentioned (105).

(f)—*2-Methyl-1, 1-dibenzylmercaptoglucose* (105).

This compound has a melting point of  $157-158^{\circ}$ . The rotation at  $25^{\circ}\text{C}$ . is  $+25.2^{\circ}$  in pyridine.

### *3-Methyl Glucose*

This sugar is mentioned but not described in 17, 27, 35, 40, 49, 50, 58, 63, 66, 81, 86, 91, 101, 102 and 105. Its reducing power has been calculated and charted (71). The alpha-modification may be obtained from the parent substances given:

(a)—*3-Methyl-1, 2, 5, 6-diacetone alpha-glucose* (3, 9, 26, 28, 32, 33, 45, 46, 48, 59, 62 and 84).

(b)—Monoacetone benzylidene glucose (38).

(c)—Hexamethyl amylobiose (69).

(d)—3-Methyl glucal (98, 101, 103).

(e)—3-Methyl alpha-methyl glucoside (69).

The methylated sugar is a definitely crystalline compound, digested by only a small number of micro-organisms (9, 57, 97). The physical constants are:

M. P.	Rotation		Solvent	Reference
157-8°	+ 98.6° → + 68.0°	(final)	methl alc.	3
157-8°	96.7° → 55.5°	"	water	3, 95
160.5°	107.6° → 68.5°	"	methl alc.	9
158°	57.0°	"	water	28
	103.0° → 57.0°	"	water	38
	104.3° → 55.3°	"	water	84
160-2°	95.0° → 52.5°	"	water	98

3-Methyl alpha-glucose crystallizes as short plates (3), readily soluble in water, less so in methyl alcohol and very sparingly in organic solvents including acetone (3, 9). It reduces Fehling's solution on warming (3). Derivatives are:

(a)—3-Methyl tetraacetyl glucose (98, 102).

Prepared by treating 3-methyl glucose with acetic anhydride in pyridine. It is a colorless syrup.

(b)—Bromo-3-methyl triacetyl glucose (98).

A colorless syrup, not crystallizable.

(c)—3-Methyl diacetyl glucal (98, 106).

A colorless syrup, b. p. 125° at 0.2 mm., showing a rotation of  $-33^\circ$  in chloroform at 20°.

(d)—3-Methyl glucal (98, 101, 103).

Long needles, m.p. 62-63°;  $[\alpha]_D^{20} + 14^\circ$  (chloroform). When this compound is treated with benzoic peracid, 3-methyl glucose is formed.

(e)—Calcium 3-methyl gluconate (97).

This calcium salt is not digested by a selected group of organisms.

(f)—3-Methyl-1, 2, 5, 6-diacetone alpha-glucose.

Prepared from 1, 2, 5, 6-diacetone glucose (3, 9, 26, 28, 32, 45, 48, 59, 76, 80, 84). It is fermentable by *B. proteus* and *B. mucosus-capsulatus* (97). The boiling point is variously stated to be 105-106° at 0.3 mm.; 139-140° at 12 mm.; and 167° at 23 mm. Refractive index of the compound is

1.4515 (9); 1.4518 (84). It is readily soluble in organic solvents, but sparingly so in water. No action on Fehling's is shown. The rotation is said to be  $-32.2^\circ$  (alc.) (3); and  $-31.4^\circ$  (84).

(g)—*3-Methyl monoacetone glucose* (76).

Prepared from (f). B.p. is  $173-175^\circ$  at 1 mm. Characterized by a *dibenzoate*, m.p.  $81^\circ$ .

(h)—*Monomethyl saccharolactone*.

On reduction, a d-3-methyl glucuronic acid is formed. This indicates that the methoxyl group in monomethyl glucose is attached to carbon three.

(i)—*Monomethyl gluconolactone* (9, 26, 48).

A syrup which decomposes easily.

(j)—*1, 4-Anhydro-3-methyl saccharic acid* (28, 38, 47).

The melting point is  $206-207^\circ$  (28, 47); with a rotation of  $+15^\circ$ .

(k)—*3-Monomethyl glucose anilide* (9).

Needles, m.p. of  $154-155^\circ$ , soluble in alcohol, sparingly so in water, insoluble in ether and hydrocarbons. The rotation is  $-108.5^\circ$  (methyl alc.)  $\rightarrow -50.3^\circ$  (final, when trace of HCl is added).

(l)—*3-Methyl alpha-methyl glucoside* (11, 83).

This glucose does not condense with acetone (9).

(m)—*Benzal-3-methyl-[methyl glucoside]* (77).

This consists of a mixture, m.p.  $133^\circ$ , showing a rotation in acetylene tetrachloride of  $+49.1^\circ$ . The *beta-form* has a melting point of  $164^\circ$ , with a rotation of  $-39.1^\circ$  in the same solvent.

(n)—*3-Methyl phenyl glucosazone*.

This is identical with the osazone obtained from monomethyl fructose derived from diacetone fructose (3, 9, 26, 32, 59, 74, 84). This osazone does not condense with benzaldehyde (9), and exhibits in alcohol the rotation (23) of  $-75.3^\circ$  (initial)  $\rightarrow -38.6^\circ$  (final) (3, 41, 95), and in pyridine  $[\alpha]_{116}$  (yellow)  $= -173^\circ (\pm 4^\circ)$  (33). The earlier declared melting point of  $164-166^\circ$  (3, 41, 95) has been shown to be incorrect (33, 84), and should be  $178-179^\circ$ .

3-Methyl alpha-glucose, when completely methylated forms the normal tetramethyl glucose ( $+83^\circ$ ) (62). It also gives rise to a d-rotatory heptonic lactone (45).

The *beta-isomer*, 3-monomethyl beta-glucose, may also be prepared from diacetone glucose (32). It is declared to show suspended rotation in methyl alcohol, and in water, with sodium light, is found  $+31.9^\circ \rightarrow +55.1^\circ$  (final) (3, 95). The sugar consists of prismatic, clear needles, soft and friable, with a melting point of  $130-2^\circ$ .



### 4-Methyl Glucose

This sugar is stated (66) to have a melting point of 156-157° (50, 95) and a rotation in water of +18.52° → +61.9° (95). The *osazone* has a melting point of 198°, and a rotation of -50.3° → -34.84° in a solution of pyridine and water (95). This monomethyl glucose should give a 1-rotatory lactone (45), and the preparation of *4-methyl glucoheptonic lactone* shows that lactones may form 4-methyl sugar acids. 2-Methyl glucose has been mistaken for 4-methyl glucose (see 55, 104, 105).

### 5-Methyl Glucose

The possibility that the monomethyl glucose from tribenzoyl alpha-methyl glucose might be this sugar is suggested (41). The *5-methyl monoacetone glucose* is said (95) to consist of long needles, with a melting point of 71-72° and a rotation of -6.42° in chloroform at 20°. The sugar itself is needle-like, m.p. 143-144°,  $[\alpha]_D +101.2^\circ \rightarrow +59.9^\circ$  (water) and soluble in water, methyl and ethyl alcohols, insoluble in ethyl acetate, benzene and petroleic ether. Fehling's solution is reduced on warming. An *osazone* of melting point 185° and rotation -101.9° → -86.6° (in pyridine) is reported (95).

### 6-Methyl Glucose

Articles which mention but do not characterize this monomethyl sugar are 9, 28, 40, 50, 65 and 72. Monomethyl trihexosan (dextrin) (65, 93) and *6-methyl alpha-methyl glucoside* (41, 61, 102) (prepared in turn from methyl tribenzoyl alpha methyl glucoside) give rise to 6-methyl glucose on hydrolysis. This compound is described as a colorless syrup (94, 102), soluble in all organic reagents, boiling at 105° at 0.1 mm., and rotating +15.3° in chloroform at 20° (94) and together with the *beta-form* in water as follows: +80.1° (init.) → +66.3° (final) (41, 95). The *6-methyl alpha-methyl glucoside* does not reduce Fehling's (41) and boils at 195-200° at 1 mm. (41, 102). The distillate has a specific rotation of +127.9° in water.

An *osazone* (54, 92) is readily obtainable, yellow needles melting at 176.9° (93); 177° (41, 95); 178° (94); 178-179° (65). All these melting points are declared to be too low (102), and the corrected one is stated to be 184-187°. The specific rotation in alcohol at various temperatures is recorded as -68.5° → -48.5° (36 hours) (65); -70.3° → -46.9° (41); -69.6° → -46.4° (94); and -70.3° → -46.9° (95).

Diacetone d-glucose dibenzyl mercaptal forms a *6-mono-methyl d-glucose dibenzyl mercaptal* (49). A 2, 3, 4-triacetyl-6-methyl beta-methyl d-glucoside, colorless crystals, m.p. 107-108°, and showing the solubilities of the simple acetyl sugars, is reported (87, 102). Its rotation at 18° is -12.4°. Emulsin fails to split *6-methyl beta-methyl d-glucoside*, a sugar soluble in water, m.p. 133-135°, and rotating -27° in water at 23° (87); -26.4° (102).

The *6-methyl-2, 3, 4-tribenzoyl beta-methyl d-glucoside* shows a melting point of 116.7° (corr.) with rotations of -118.4° (pyridine) and -54° in chloroform (102). A number of tetraacetyl derivatives are reported (102). There is a *tetraacetyl-6-methyl-alpha-d-glucose* of m.p. 119-120° and exhibiting a rotation of +111.8°; and the corresponding *beta* derivative of m.p. 91-93° with a rotation of -20.9° at 22°. The latter may give rise to a 2, 3, 4-triacetyl 6-methyl glucosyl bromide.

## DI-ALKYL GLUCOSSES

Completely methylated cellulose should not give dimethyl glucose (42, 75), but a dimethyl methyl glucoside and, in turn, by the hydrolysis of the glycoside group, a dimethyl glucose has been reported (7, 18, 44, 75, 82) as being obtained from a methylated cellulose with as high a methoxyl content as 43.1 per cent (44). There is probably some trimethyl glucose mixed with the dialkylated glucose (44, 82). The mixture is a golden yellow syrup of  $n_D = 1.4632$  (82), giving an amorphous dimethyl glucose, and forming an *osazone* (7). An amorphous compound is mentioned in several (15, 30) other papers. Likewise, incompletely methylated starch gives some dimethyl glucoside (78).

Dimethyl glucoses are mentioned in 1, 2, 7, 9, 15, 23, 24, 30, 31, 35, 36, 37, 66, 73 and 77; a dimethyl glucoside in 23, 24; and a *diethyl glucose* in 15 and 73. A dimethyl glucose, structure not given, was found to be completely stable to fuming HCl. Monomethyl anhydro-methyl glucoside (43) forms a *dimethyl anhydro-methyl glucoside*. The possibility of the existence of 3, 4-dimethyl glucose is upheld by Irvine (24, 25) and co-workers. Polymerized glucosan is said to give an unidentified dimethyl methyl glucoside and dimethyl glucose (52). Other compounds found reported in the literature are a *dimethyl monoacetyl glucose* (51), and the *hexa-acetate of the dimethyl glucoside of bis-[glucosyl-6] sulfone* (56).

2, 3-Dimethyl  $\alpha$ -Glucose

This interesting carbohydrate derivative may be directly obtained from the methylated galaetosido-glucose of Fischer (70) or from the glucoside (4, 6, 8, 10, 22, 24, 25, 39, 64, 83, 88, 89). Mention of the sugar is made in 1, 60, 105. It is seen that entire agreement is lacking as to its physical properties:

M. P.	Rotation	Solvent	References
	+ 81.93°	Water	3
85.87°	81.93° → 48.3°	"	4
Syrup	65.30° → 64.4°	"	6
	57.20° → 58.1°	Methyl alc.	6
	50.00° → 49.1°	Ethyl alc.	6
	43.7°	One equiv. NaOH	29, 67
	22.5°	Neut. sol'n	29, 67
85-87°	64.4°	Water	47
156-7°	93.10° → 62.4°	"	60
	110.00° → 64.7°	Methyl alc.	60
	50.3°	Acetone	64
	83.85° → 85.07°	Water	83
Syrup	57.8°	Acetone	89

The sugar is stable toward potassium permanganate, readily soluble in water, lower alcohols and acetone, sparingly in ethyl acetate, insoluble in ether and hydrocarbons. The reducing power has been calculated (71).

Derivatives are:

(a)—2, 3-Dimethyl gluconic acid lactone (53, 67).

(b)—Crystalline tetramethyl glucose (24, 25, 60).

(c)—2, 3-Dimethyl saccharic acid (47).

Rotation = + 55.4° (water); + 80.15° (acetone).

(d)—2, 3-Dimethyl gluconic acid and Na salt (53, 67, 68).

The acid forms lactones with five and six-membered rings. A brown syrup is secured when 2, 3-dimethyl glucose is heated with concentrated nitric acid at 80° (83).

(e)—Dimethoxyglucosephenylhydrazone (4, 47, 60, 62, 89).

This is a pale yellow syrup. No osazone is formed.

(f)—2, 3-Dimethyl-5, 6-l-benzylidene  $\alpha$ -methyl d-glucoside (4, 53, 60, 67, 77).

The melting point is 117-119° (60); 122-123° (4, 53, 67, 77); the rotation is + 87.03° (acetone) (4). It crystallizes in the form of prisms, sparingly soluble in water but readily so in organic solvents.

(g)—Beta derivative of (f) (77).

The melting point is 134°, and the sugar exhibits a rotation of — 61° in alcohol at 23°C.

(h)—2, 3-Dimethyl  $\alpha$ -methyl glucoside (23, 24).

Can be obtained from (f) (4); glucogen (47); glucose (8, 10, 60, 89); incompletely methylated starch (22, 24, 25, 39, 64, 83, 88) and from 2, 3-dimethyl methyl glucoside 5, 6-monoacetone (6, 26). It does not condense with acetone (60) and has a refractive index of 1.4738 (89). Other constants are: m.p. 80-82°; + 142.6° (water) (4), + 143.08° (alc.) (4, 19), and + 143.49° (acetone) (4, 47).

(i)—2, 3-dimethyl methyl glucoside 5, 6-monoacetone (26).

$[\alpha]_D^{25} = -3.76^\circ$  (methyl alcohol) (80).

### 2, 3-Dimethyl $\beta$ -Glucose

Mentioned only in 1, 3 and 60. Acetone solutions of the compound are stable (12). The melting point is 108-110° (4, 47) and the mutarotation is upward. The specific rotation is given (4) as:

+ 10.6°	→	64.4°	(water-final)
+ 5.68°	→	49.41°	(alcohol-final)
+ 6.52°	→	50.9°	(acetone-final)

## REFERENCES

1912

1. Irvine, J. C.—*Procs. Chem. Soc.*, (London) 29:69.
2. Irvine, J. C.—*Ann. Reports Prog. Chem.*, 10:77-89.
3. Irvine, J. C., and J. P. Scott—*J. Chem. Soc.*, (London) 103:564-575.
4. Irvine, J. C., and J. P. Scott—*J. Chem. Soc.*, (London) 103:575-586.
5. Irvine, J. C., R. F. Thomson and C. S. Garrett—*J. Chem. Soc.*, (London) 103:238-249.
6. MacDonald, J. L. A.—*J. Chem. Soc.*, (London) 103:1896-1904.

1914

7. Denham, W. S., and H. Woodhouse—*J. Chem. Soc.*, (London) 105:2357-2368.
8. Haworth, W. N.—*Procs. Chem. Soc.*, (London) 30:293-294.
9. Irvine, J. C., and T. P. Hogg—*J. Chem. Soc.*, (London) 105:1386-1396.

1915

10. Haworth, W. N.—*J. Chem. Soc.*, (London) 107:8-16.
11. Irvine, J. C., and J. L. A. MacDonald—*J. Chem. Soc.*, (London) 107:1701-1710.
12. Irvine, J. C., and E. S. Steele—*J. Chem. Soc.*, (London) 107:1230-1240.

1920

13. Pictet, A., and P. Castan—*Helv. Chim. Acta*, 3:645-649.
14. Pictet, A., and P. Castan—*Compt. rend. Acad. Sci.*, 171:243-245.
15. Hess, K., W. Wittelsbach and E. Messmer—*Zeits. angew. Chem.*, 34:449-454.
16. Irvine, J. C., and J. W. H. Oldham—*J. Chem. Soc.*, (London) 119:1744-1749.
17. Karrer, P., and O. Hurwitz—*Helv. Chim. Acta*, 4:728-734.
18. Reilly, J.—*Helv. Chim. Acta*, 4:616-621.

1922

19. Carruthers, A., and E. L. Hirst—*J. Chem. Soc.*, (London) 121:2299-2308.
20. Cramer, M., and E. H. Cox—*Helv. Chim. Acta*, 5:884.
21. Gilchrist, H. S.—*J. Soc. Chem. Ind.*, 41:365R.
22. Haworth, W. N.—*Ann. Reports Prog. Chem.*, 19:77.
23. Irvine, J. C., and co-workers—*Brit. Ass'n Reports, Chem. News* 125:165-170; 181-186.
24. Irvine, J. C., and co-workers—*J. Soc. Chem. Ind.*, 41:362-365R.
25. Irvine, J. C.—*Report Brit. Ass'n Sci.*, 90:33-48.
26. Irvine, J. C., and J. Patterson—*J. Chem. Soc.*, (London) 121:2146-2161.
27. Irvine, J. C., E. S. Steele and M. I. Shannon—*J. Chem. Soc.*, (London) 121:1060-1078.
28. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, 54:805-813.
29. Macbeth, A. K., and J. Pryde—*J. Chem. Soc.*, (London) 121:1660-1668.
30. Pringsheim, H., and K. Schmalz—*Ber.*, 55:3001-3007.

1923

31. Bridel, M.—*Bull. Soc. Chim.*, 33:1005-1058.
32. Freudenberg, K., and A. Doser—*Ber.*, 55:1243-1247.
33. Freudenberg, K., and R. M. Hixon—*Ber.*, 56:2119-2127.
34. Hirst, E. L., and D. R. Morrison—*J. Chem. Soc.*, (London) 123:1352-1360.
35. Irvine, J. C.—*J. Chem. Soc.*, (London) 123:898-921.
36. Irvine, J. C., and E. L. Hirst—*J. Chem. Soc.*, (London) 123:518-532.
37. Karrer, P.—*Helv. Chim. Acta*, 6:402-409.
38. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, 57:319-322.
39. Ling, A. R., and D. R. Nanji—*J. Chem. Soc.*, (London) 123:2636-2688.

1924

40. Haworth, W. N.—*Ann. Reports Prog. Chem.*, 21:66.
41. Helferich, B., and J. Becker—*Annalen*, 440:1-18.
42. Hess, K.—*Zeits. angew. Chem.*, 37:993.
43. Irvine, J. C., and H. S. Gilchrist—*J. Chem. Soc.*, (London) 125:1-10.
44. Karrer, P., and K. Nishida—*Helv. Chim. Acta*, 7:363.
45. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, 60:173-178.
46. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, 59:145-149.
47. Macbeth, A. K., and J. Mackay—*J. Chem. Soc.*, (London) 125:513-521.



48. Ohle, H.—Ber., 57:403-409.  
49. Pacsu, E.—Ber., 57:849-853.

## 1925

50. Dorée, C.—Ann. Reports Prog. Chem., 22:82-103.  
51. Heuser, E., and N. Hiemer—Cellulosechemie, 6:101-122; 125-132; 153-166.  
52. Irvine, J. C., and J. W. H. Oldham—J. Chem. Soc., (London) 127:2903-2922.  
53. Levene, P. A., and G. M. Meyer—J. Biol. Chem., 65:535.  
54. Ohle, H.—Ber., 58:2577-2584.  
55. Pacsu, E.—Ber., 58:1455.  
56. Wrede, F., and W. Zimmerman—Z. physiol. Chem., 148:65-82.

## 1926

57. Coles, H. W.—Plant Physiology, 1:379-385.  
58. Freudenberg, K., and K. Smeykal—Ber., 59:100-107.  
59. Haworth, W. N.—Ann. Reports Prog. Chem., 23:74-97.  
60. Haworth, W. N., and W. G. Sedgwick—J. Chem. Soc., (London) 129:2573-2580.  
61. Helferich, B., W. Klein and W. Schäfer—Ber., 59:79.  
62. Hudson, C. S.—J. Amer. Chem. Soc., 48:1434.  
63. Irvine, J. C., and I. M. Black—J. Chem. Soc., (London) 129:862.  
64. Irvine, J. C., and J. MacDonald—J. Chem. Soc., (London) 129:1502-1518.  
65. Kuhn, R., and W. Ziese—Ber., 59:2314-2316.  
66. Leibowitz, J.—Zeits. angew. Chem., 39:1143-1148; 1240-1249.  
67. Levene, P. A., and G. M. Meyer—J. Biol. Chem., 70:343.  
68. Levene, P. A., and H. S. Simms—J. Biol. Chem., 68:737-749.  
69. Pringsheim, H., and A. Steingrover—Ber., 59:1001-1006.  
70. Schlubach, H. H., and W. Rauchenberger—Ber., 59:2102-2106.  
71. Sobotka, H.—J. Biol. Chem., 69:267-275.

## 1927

72. Gray, H. LeB., and C. J. Staub—Chem. Reviews, 4:355-373.  
73. Hess, K., W. Wittelsbach and E. Messmer—Zeits. angew. Chem., 34:449-454.  
74. Ohle, H.—Ber., 60:1168-1174.

## 1928

75. Freudenberg, K., and E. Braun—Annalen, 460:288-304.  
76. Freudenberg, K., W. Durr and H. v. Hochstetter—Ber., 61:1735-1743.  
77. Freudenberg, K., H. Toepffer and C. C. Andersen—Ber., 61:750-760.  
78. Haworth, W. N., E. L. Hirst and J. I. Webb—J. Chem. Soc., (London) 2681-2690.  
79. Hickenbottom, W. J.—J. Chem. Soc., (London) 3140-3147.  
80. Levene, P. A., and G. M. Meyer—J. Biol. Chem., 79:357-362.  
81. Montonna, R. E.—Paper Trade J., No. 18, 86:61-70.  
82. Rigby, G. W.—J. Amer. Chem. Soc., 50:3364-3370.  
83. Schmid, L., and M. Zentner—Monatsh., 49:111-117.

## 1929

84. Anderson, C. G., W. Charlton and W. N. Haworth—J. Chem. Soc., (London) 1329-1337.  
85. Brigl, P., and R. Schinle—Ber., 62:1716-1723.  
86. Freudenberg, K., W. Belz and C. Niemann—Ber., 62:1554-1561.  
87. Helferich, B., and E. Himmen—Ber., 62:2136-2141.  
88. Irvine, J. C.—Rec. trav. chim., 48:813-816.  
89. Irvine, J. C., H. Pringsheim and A. F. Skinner—Ber., 62:2372-2778.  
90. Josephson, K.—Ber., 62:313-316.  
91. Josephson, K.—Annalen, 472:230-240.  
92. Lieser, T.—Annalen, 470:104-110.  
93. Link, K. P.—J. Amer. Chem. Soc., 51:2516-2522.  
94. Ohle, H., and L. v. Vargha—Ber., 62:2425-2434.  
95. Ohle, H., and L. v. Vargha—Ber., 62:2435-2444.  
96. Brigl, P., and R. Schinle—Ber., 63:2884-2887.  
97. Kendall, A. L., and C. E. Gross—J. Infect. Diseases, 47:249-260.  
98. Levene, P. A., and A. L. Raymond—J. Biol. Chem., 88:513-518.  
99. Papadakis, P. E.—J. Amer. Chem. Soc., 52:2147.

- 100. Papadakis, P. E.—J. Amer. Chem. Soc., **52**:3465.
- 101. Farmer, E. H.—Ann. Reports Prog. Chem., **27**:102-114.

## 1931

- 102. Helferich, B., and E. Gunther—Ber., **64**:1276-1280.
- 103. Hirst, E. L., and C. S. Woolvin—J. Chem. Soc., (London) 1131-1137.
- 104. Levene, P. A., G. M. Meyer and A. L. Raymond—Science, **73**:291-292.
- 105. Levene, P. A., G. M. Meyer and A. L. Raymond—J. Biol. Chem., **91**:497-504.
- 106. Levene, P. A., and A. L. Raymond—J. Biol. Chem. **90**:247-250.
- 107. Helferich, B., and H. Appel—Ber., **64**:1841-1847.

## THE LITERATURE OF ALKYLATED CARBOHYDRATES

### VI. TRI-, TETRA-, AND PENTA-ALKYLATED GLUCOSE DERIVATIVES

HAROLD W. COLES

*From the Laboratory of Plant Chemistry, Department of Chemistry, Iowa State College*

Accepted for publication August 12, 1931

#### TRI-ALKYLATED GLUCOSES

A large number of references to trimethyl glucoses may be found in the literature without any definite mention as to the position of the alkyl groups. These are recorded here for the purpose of giving those interested a complete list of references.

When diethyl mercapto-glucose is methylated (153) a *trimethyl* derivative is obtained. Cellotriose can be hydrolyzed (252) with the formation of a *tri-(trimethyl [1, 5-anhydro-glucose])*. Glucose, on treatment with dimethyl sulfate, gives rise to a *trimethyl methyl glucoside*, the melting point of which is said to be 64° (140). A trimethyl glucose is secured (96) from the partial hydrolysis of *trimethyl methyl glucoside mono-oleate*. An unknown trimethyl gamma-sugar is secured (12), together with 2, 3, 6-trimethyl glucose, from the galactosido-glucose of Fischer and Armstrong.

Interesting derivatives are obtained when beta-glucochloralose is methylated (229). A *trimethyl beta-glucochloralose*, of melting point 109°-110° is secured. This gives a Zerewitinoff test and forms a *monoacetyl* derivative. On removal of one chlorine group, *trimethyl-monodechloro-beta-glucochloralose* (trimethyl glucose dichloro-acetaldehyde) with a melting point of 68° is secured. When two chlorine groups are taken off, a *bidechloralose* compound is secured which is a syrup, of b.p. 155°-160° at 4 mm. A *trimethyl saccharolactone* is mentioned (148).

#### 2, 4, 6-Trimethyl Glucose

This sugar is mentioned in 107, 152 and 169. The methyl groups present in the trimethyl glucose secured from heptamethyl sucrose was early tentatively (140) assigned to the 2, 4 and 6 carbon atoms, but was later shown to be 2, 3 and 6. The 2, 4, 6-trimethyl glucose is reported (211) as having a melting point of 124°.

#### 3, 4, 6-Trimethyl Glucose

Leibowitz (152) mentions this compound. Although little is said about the sugar itself, considerable work has been reported on its derivatives:

(a) *3, 4, 6-Trimethyl glucal* (271, 285).

This is a colorless mobile oil, b.p. 45°/0.3 mm., with a refractive index of 1.4558 and a rotation of  $[\alpha]_D^{18} = +19.6^\circ$  (water). The unsaturated

properties of glucal are retained as well as the double bond between the first and second carbon items. It is unstable in hot, alkaline solutions, giving dark-colored decomposition products. In addition to normal trimethyl glucal, some *trimethyl pseudo-glucal* appears to be present. The glucal gives trimethyl glucose on treatment with perbenzoic acid which, on methylation, forms the 2, 3, 4, 6-tetramethyl glucopyranose.

On treatment with aqueous mineral acid there is secured:

(b) *Trimethyl-2-deoxyglucose* (271, 285).

Also called 3, 4, 6-trimethyl-d-glucodesose. It is a pale yellow syrup, b.p. 102-105° at 0.01 mm.,  $n_D^{18} = 1.4622$ . In crystalline form it melts at 59-60° (285); 58-61° (271), and the initial rotation at 18° is +61° (5 mins.)  $\rightarrow$  +35° (3 hrs.) (285); +59.5° (water)  $\rightarrow$  +33.1° (equil.) (271).

(c) *Barium 3, 4, 6-trimethyl d-glucodesonate* (271).

$[\alpha]_D^{25} = +18.1^\circ$  (water).

(d) *Trimethyl 2-deoxygluconolactone* <1, 5> (271, 285).

Colorless oil, b.p. 120°/0.04 mm.;  $n_D^{17} = 1.4606$ . The rotation at 25° in chloroform is 87.5° and in benzene is 88.2°.

(e) *Trimethyl d-methyl glucodesoside* (271).

The boiling point at 0.35 mm. is 86-90°. It is prepared from methyl-2-deoxy glucoside.

(f) *Trimethyl d-glucodesonic lactone* <1, 4> (271).

Prepared from glucodesonic lactone. It has a melting point of 62° (uncorr.) and a rotation of 21.5° in benzene at 25°.

(g) *Trimethyl-2-deoxy gluconic acid phenylhydrazide*.

Colorless silky needles (285), m.p. 25°.

(h) *Trimethyl gluconic acid phenylhydrazide* (236).

Melting point is 125°.

### 3, 4, 5-Trimethyl Glucose

The 3, 4, 5-trimethyl methyl glucoside is reported (138) to give, when treated with concentrated nitric acid( d-arabotrimethoxyglutaric acid, identified as amide, and methyl dl-dimethoxysuccinate, m.p. 67-68°, identified as the amide, having a melting point of 245-246° (decomp'n).

### 3, 5, 6-Trimethyl Glucose

This sugar is casually mentioned without any description of its physical and chemical properties being given in 29, 52, 55, 107, 108, 138-140, 152, 228 and 287. Its reducing power has been determined (166, 287). The mother compound of this sugar is 3, 5, 6-trimethyl-1, 2-monoacetone glucose <1, 4>, which is prepared by methylation of 1, 2-monoacetone glucose (1, 2, 15, 24, 25, 50, 64, 102, 116, 160, 187, 249).



The *3, 5, 6-trimethyl-1, 2-monoacetone glucose* is so important that it is necessary to record its characteristics in the following table:

B. P.	Pressure	Rotation	Solvent	Reference
138-9°	12 mm.			15
88-90°	0.03	— 28.5°	Water	50
110°	0.30			116
115-8°	0.60			154
110-5°	0.20	— 27.2°	Methyl alc.	160
		— 29.5°	" "	228
96°	0.05	— 27.2°	" "	249

The compound is a syrup which is stated by one worker to reduce Fehling's in the cold (187), while another (154) claims that it doesn't have that property. A refractive index is recorded of 1.4491 (116) and 1.4470 (228).

The trimethyl glucose is obtained from the methylated monoacetone glucose by removal of the acetone residue (1, 15, 25, 50, 64, 116, 154, 160, 187, 228 and 249). The following constants are recorded in the literature for this important furanose sugar:

B. P.	Pressure	Rotation	Solvent	Reference
		— 37°		1, 154
		— 8° to — 11°	Water	1
		— 8.32°	"	15
		— 6.2° → — 8.3°	"	15
147°	0.05 mm.	— 10.95° → — 14.6°	"	50
153°	0.15	— 37.3°	Alcohol	64
		— 8° → — 11°	Water	155, 187
		— 31.1°	Alcohol	160
		— 12.4° → — 10°	"	187
150°	0.04	— 44.1°	"	228
		— 25.9°	Water	228
		+ 2.6°	"	249

It is a colorless liquid (15, 50, 64, 228), reducing neutral permanganate rapidly (64), with a refractive index of 1.4675 (228), and acting very much like a trimethyl glucosone on distillation (25). Although it is generally agreed that the substance is levo-rotatory (81, 102, 154), the *diacetal* is the opposite (154). When pure, the trimethyl glucose is said not to reduce Fehling's solution in the cold. It condenses with orcinol-HCl to form a deep-blue pigment (154, 155).

Derivatives of this sugar are:

(a) *Trimethyl gluconic acid* (154, 155, 187).

It rotates + 24° in on equivalent of NaOH and when this is neutralized, the sign changes and the constant becomes — 6.35°. The *sodium salt* has a rotation of + 31°.

(b) *Trimethyl phenylglucosazone*.

Melting point is 70-72° (228); 163-164° (55).

(c) *Trimethyl gluconolactone* (116).

(d) *3, 5, 6-Trimethyl glucosone* (25).

A syrup, b.p.  $153^{\circ}/0.15$  mm. It reacts with Fehling's.  $[\alpha]_D = -37.3^{\circ}$  (alc.);  $-15.7^{\circ}$  (water); and  $-15.7^{\circ}$  (N/4 boric acid).

(e) *3, 5, 6-Trimethyl beta-glucochloralose* (229).

This substance has already been discussed.

(f) *Ba salt of 3, 5, 6-trimethyl-2-phosphoric acid methyl glucoside* (2, 50).

$[\alpha]_D^{20} + 26.38^{\circ}$ . Does not reduce Fehling's until after hydrolysis with acid.

(g) *Trimethyl methyl glucoside* (50).

The mixture of alpha- and beta-glucosides is a colorless syrup,  $n_D^{16} = 1.4440$  (228);  $[\alpha]_D^{16} = -20.4^{\circ}$  (water) (228), although the rotation is stated to be  $+21.4^{\circ}$  by 187. This mixture can be fractionated into a lower fraction, the alpha, of b.p.  $105-109^{\circ}/0.4$  mm., rotation  $+93^{\circ}$  (187);  $+50^{\circ}$  (water) (1, 154, 155);  $+75^{\circ}$  (methyl alc.) (2); and a higher fraction, the beta-glucoside, b.p.  $145-150^{\circ}$  at 0.2 mm.,  $[\alpha]_D^{20} = -87^{\circ}$  (water) (187);  $-17^{\circ}$  to  $-50^{\circ}$  (water) (1, 154, 155);  $-47^{\circ}$  (methyl alc.) (2).

On methylation, the trimethyl glucoside forms the well-known 2, 3, 5, 6-tetramethyl methyl glucoside.

### *2, 3, 5-Trimethyl Glucose.*

Under this title will also be given the literature on 2, 3, 4-trimethyl glucose, which is wrongly numbered. Papers which mention this compound without specific description are: 6, 20, 28, 34, 41, 56, 59-61, 65, 73, 75, 78, 82, 86, 94, 98, 100, 107, 109, 119, 133, 138, 144, 147-149, 152, 158, 160, 163, 174, 181-184, 196, 224.

Many ways are available for preparing this trimethyl glucose. They are:

(a) Methylated gentiobiose (105, 108, 201).

(b) Methylated melibiose (127, 169, 176).

(c) Hendecamethyl raffinose (74, 75, 169, 201).

(d) Methylated amygdalin (57, 58, 74, 75, 85, 93, 108, 147).

(e) Methyl glucoside (1, 2, 35, 152, 155).

(f) Trimethyl l-glucosan (66, 105, 109, 123, 161, 244).

(g) Polymerized glucosan (114).

(h) Beta-glucosan (114).

(i) Methylated gluco-mannan (273).

The trimethyl glucose is a syrup (15, 108) distilling at  $194^{\circ}$  at 9 mm., soluble in water and in organic solvents but not in ether (1). It is completely

stable to fuming HCl (80). Its reducing value is 9.8 if glucose is given a value of 100 (130). It is said (91) to have no effect in relieving the symptoms caused by insulin in mice. In one equivalent of NaOH, the rotation is  $+64.4^\circ$  (116) and when the same solution is neutralized it is found to be  $+19.3^\circ$ . Other values given are  $+79.2^\circ$  (1);  $+68.7^\circ$  (114); and  $+67.8^\circ$  (127), in every case methyl alcohol being the solvent.

Derivatives of this important sugar are:

(a) *2, 3, 5-Trimethyl saccharic acid lactone*.

Mentioned in 1, 58, 63, 108, 116, 244 and 273. Prepared by oxidation of the trimethyl glucose with concentrated nitric acid. When the glucoside from "Konjak" is hydrolyzed and oxidized this compound is secured. The saccharic acid itself could not be isolated (1).

(b) *1, 6-Diacetyl-2, 3, 5-trimethyl glucose* (109, 201).

This forms a syrup which does not crystallize when oxidized with fuming nitric acid.

(c) *Trimethyl-6-phosphoric acid methyl glucoside* (50).

A white hygroscopic powder, very soluble in alcohol and acetone, with  $[\alpha]_D + 77.07^\circ$  in water.

(d) *Trimethyl acetyl glucose bromohydrin* (109).

(e) *Trimethoxyaraboglutaric acid* (152).

Formed by the degradative oxidation of the trimethyl glucose with nitric acid.

(f) *Trimethyl gluconic acid* (154).

$[\alpha]_D + 64.4^\circ$  (one equiv. NaOH)  $\rightarrow + 38.6^\circ$  (neut.).

(g) *Trimethyl glucose dinitrate* (123).

Prepared by treating trimethyl l-glucosan in chloroform with fuming nitric acid. Has no action on Fehling's before or after hydrolysis.

(h) *Trimethyl methyl glucoside 6-mononitrate*.

Prepared from (g).

(i) *Trimethyl methyl glucoside iodohydrin* (113, 123).

Prepared from (h) or from acetodibromoglucose.

(j) *Trimethyl beta-methyl d-glucoside*.

Described in 35, 58, 59, 74, 75, 108, 114, 132, 135, 187, 213. It may be prepared from triphenyl methyl glucose (174), from (d), from methylation of methyl glucoside or, as usually done, from the parent sugar itself. It is a crystalline compound, melting at  $92.5^\circ$  (108);  $92.93^\circ$  (174);  $93.94^\circ$  (149, 169);  $94^\circ$  (59). The rotation is recorded as  $-22.9^\circ$  (54);  $-23.1^\circ$  (methyl alcohol) (149); and  $-25.1^\circ$  (water) (108). As a syrup it distills at  $108^\circ/0.07$  mm. (50) or  $109^\circ/0.06$  mm. (58). It is soluble in ether (1),

has a refractive index of 1.4579, and a density of 1.1477 at 20° (50). The glucoside was not digested by members of the colon-typhoid group, or organism isolated from the sludge of creamery wastes (132). Its reducing value is 9.4 where glucose is 100.

(k) *Trimethyl alpha-methyl d-glucoside.*

From the parent sugar (123) or from (h). A viscid syrup (2, 19, 22), distilling at 130°/0.13 mm. with  $n_D = 1.4583$  and  $d_4^{20} = 1.158$ .

*2, 3, 6-Trimethyl Glucose*

This trimethyl glucose results as the scission product of many compound sugars and, consequently, more has been written about this sugar than any of the others already discussed. This sugar is mentioned, but not specifically described in 6, 20, 28, 30, 32, 38, 39, 41, 46, 52, 56, 59, 60, 65, 66, 68, 73, 94, 98, 103, 106, 107, 109, 111, 119, 128, 129, 136, 144, 152, 158, 160, 163, 167, 181-184, 196, 223, 238, 263, 273.

Its sources of preparation are:

- (a) Methylated hexa-amylose (114, 290).
- (b) Heptamethyl sucrose (36, 77, 108, 140, 219).
- (c) Methylated lichenin (100, 124).
- (d) Trimethyl starch (57, 60, 61, 82, 84, 86, 138, 149, 182, 211, 239, 248, 290).
- (e) Hexamethyl biosan (143).
- (f) Decamethyl  $\beta$ -methyl cellotrioside (284).
- (g) Octamethyl cellobiose (45, 47, 48, 49, 63, 82, 108, 143, 209, 233, 280).
- (h) Octamethyl maltose (33, 35, 82, 108, 133, 138, 148, 176, 201, 202, 209, 233).
- (i) Trimethyl glycogen (101, 182, 234).
- (j) Octamethyl lactose (30-32, 45, 59, 60, 63, 82, 89, 111, 127, 159, 164, 167, 177, 209, 219, 233).
- (k) Trimethyl cellulose (29, 31, 38-40, 42, 44, 45, 53, 60-63, 66, 79, 83, 92, 97, 100, 109, 112, 114, 136, 138, 143, 149, 159, 207, 210, 219, 220, 224, 225, 239, 251, 283, 290).
- (l) Methylated galactosido-glucose (165).

The 2, 3, 6-trimethyl glucose is said to exist (239) in two interconvertible forms, the butylene and amylenes. It is stable to fuming HCl and is sparingly soluble in dry ether. It is stable to potassium permanganate (31, 33), reduces copper solutions (33), forms no osazone (18, 33, 108, 140, 146), and does not undergo inversion of sign during condensation with methyl alcohol and HCl at room temperature (140). Tetramethyl glucose can be easily separated from the trimethyl glucose by extracting the former with boiling light petroleum (63). The x-ray picture of this compound has been studied (216, 231).



Other physical characteristics of this sugar are:

M. P.	Rotation	Solvent	Reference
110°	+ 101.12° → + 69.52°	Acetone	18
	103.96° → 67.49°	Water	18
118-9°	108.00° → 67.00°	"	31
	67.80°	Methyl alc.	33
	69.30° → 71.00°	Water	33
115°	104.20° → 69.00°		48
	108.00° → 67.00°	"	60, 61
	117.70° → 88.60°	Methyl alc.	63
	90.20° → 70.50°	Water	63
	117.70° → 70.00°	HCl-hr.	63
94-104°			83
116°			100
107-110°	75.00° → 67.84°	Water-5 days	101
	71.00°	Water-final	108
	54.10°	CH <sub>3</sub> OH-HCl	112
	86.30°	60 hrs. in bomb	112
	88.60°	Methyl alc.	127
109-112°	90.60° → 70.11°	Water	143
113-4°	70.5°	Water-final	148
105°			150
103.7°	70.2°		193
100-3°	70.01°	Water-final	194
	69.20°	" "	194
114-5°	90.7° → 70.20°	" "	207
117-8°			211
118°	67°	" "	234
110-112°			241
114-5°			279

Two crystal forms have been isolated (63)—fine needles, of melting point 122-123° and short prisms melting at 92-93°. The boiling point of the substance as a syrup has been variously stated as 138-150°/0.04 mm. (89); 170°/0.43 mm. (108); and 171° at 0.23 mm. The refractive index is 1.4743 (63) and 1.4745 (89). The reducing value is 27.1 if glucose is assigned a value of 100. When treated with phosphorus pentachloride, crystals melting at 160° are obtained (207).

Derivatives are:

(a) *Trimethyl d-glucose dibenzyl mercaptal* (103).

(b) *Trimethyl-1, 4-diacetyl beta-glucose* <1, 5> (194, 214).

Melting point 67-68°;  $[\alpha]_D^{19} = -8.7^\circ$  (chloroform).

(c) *Trimethyl-1-chloro-4 acetyl α-glucose* (194).

Distills undecomposed at 143-146°/0.04 mm.  $[\alpha]_D^{26} = +143^\circ$ .

(d) [2, 3, 6-Trimethyl-4-acetyl glucosido <1, 5>]-trimethyl ammonium chloride (194).

Rotation at  $19^\circ$  is  $-3.6^\circ$  (chloroform).

(e) [2, 3, 6-Trimethyl  $\alpha$ -glucosido  $<1, 4>$ ]-trimethyl ammonium hydroxide (214).

Fine needles, melting at  $187-188^\circ$ , with a rotation at  $21^\circ$  of  $-68.3^\circ$  (water). Heating in a vacuum produces a brownish-yellow oil.

(f) [Trimethyl  $\alpha$ -glucosido  $<1, 4>$ ]-trimethyl ammonium chloride (214).

M.p.  $165^\circ$ .  $[\alpha]_D^{17} = -68.4^\circ$  (water).

(g) [2, 3, 6-Trimethyl-5-benzoyl glucosido  $<1, 4>$ ]-trimethyl ammonium chloride (214).

Prepared by benzylation in pyridine of (f). Consists of compact prisms, of melting point  $102-104^\circ$ . The substance is a molecular compound of one mol with one mol of pyridine-HCl.  $[\alpha]_D^{20} = -47.1^\circ$  (water); m.p.  $146-149^\circ$ .

(h) [2, 3, 6-Trimethyl-5-acetyl-glucosido  $<1, 4>$ ]-trimethyl ammonium chloride (214).

Is amorphous and does not form an anhydride.

(i) Dimethyl saccharic acid and lead salt (63).

(j) Trimethyl glucoheptonic acid (29, 31, 63).

Forms a dimethyl gluco-heptonolactone.

(k) 1-Dimethyl-amino-2, 3, 6-trimethyl glucose.

Melts at  $123^\circ$  (140, 207), and distils at  $109^\circ/0.01$  mm. The rotation is recorded as  $+18.6^\circ$  (water),  $+7.2^\circ$  (methyl alc.) (207);  $+89.7^\circ \rightarrow -71.9^\circ$  (water);  $+110.0^\circ \rightarrow 69.7^\circ$  (methyl alc.) (140).

(l) 1 (or 4?)-Chloro-2, 3, 6-trimethyl glucose.

(Described in 207, 210.) A colorless syrup, b.p.  $140-150^\circ/0.1$  mm., easily soluble in petroleic ether, but with a limited solubility in water. It readily reduces Fehling's solution and is decomposed by water. A crystalline pyridinium salt of rotation  $+26.6^\circ$  in water is formed.  $[\alpha]_D^{20} = +27.5^\circ$  (in chloroform).

(m) Quaternary ammonium iodide compound (207).

Prepared from (k). Needles of  $-41.2^\circ$  (water).

(n) Quaternary ammonium chloride compound (207).

Prepared from (k). Needles rotating  $-9.4^\circ$  in water at  $16^\circ$ .

(o) 2, 3, 6-Trimethyl-5-benzoyl-beta-methyl glucoside (89, 214).

Boiling point is  $134-135^\circ/0.08$  mm.,  $n_D^{20} = 1.5020-1.5028$ . Rotations are given as  $[\alpha]_D^{18} = -23.47^\circ$  (50% alc.);  $[\alpha]_D^{20} = -39.5^\circ$  (methyl alc.).

(p) 2, 3, 6-Trimethyl-5-acetyl- $\beta$ -methyl glucoside.

$n_D = 1.4470$  (89).

(q) *2, 3, 6-Trimethyl-5-benzoyl-1 chloroglucose* <1, 4>.

(r) *Trimethyl saccharomonolactone* (33, 35, 108, 133).

This pale yellow syrup distils at 152-155° (35), and rotates + 40° in water after 4 hours.

(s) *Trimethyl gluconic acid* (137, 145, 155).

(t) *Trimethyl gamma-lactone* <1, 4> (137, 139, 145, 155).

(u) *2, 3, 6-Trimethyl  $\alpha$ -methyl glucoside* (150, 187, 207).

This may be prepared either from the trimethyl glucose (29, 60, 61, 62, 98, 100, 106, 112, 217, 280); from the octamethyl lactose (89); or from trimethyl glycogen (101, 182, 234). When combined with 1-chloro-2, 3, 4, 6-tetramethyl glucose, octamethyl cellobiose may be synthesized (259). The boiling point is 95-97°/0.02 mm. (211); 100°/0.03 mm. (234); and the refractive index is 1.4550 at 24° (211). When combined with the beta-isomer,  $n_D = 1.4583$  (224); b.p. = 118-120°/1 mm. (143) and 147-150°/0.07 mm. (63), and the rotations vary as given: - 30° (in methyl alc.); + 62.04° (methyl alc.) (143); + 66.1° (224); + 66.5° (alc.) (63), and + 87.2° (159). When treated with  $PCl_5$  (207) the glucoside forms a colorless syrup, b.p. 88-95°/0.1 mm., with a limited solubility in water and having the composition of a chlorohydrin.

(v) *2, 3, 6-Trimethyl  $\beta$ -methyl glucoside* (138).

May be prepared from the trimethyl glucose itself (59, 60, 109, 260) or from tetramethyl glucose-1-chlorohydrin (260). It can be separated from galaetosides, for example when methylated lactose is hydrolyzed, by benzoylating and fractionally distilling (89). The physical constants are:

M.P.	B.P.	Rotation	Colvent	Reference
60.5°	81°/.04 mm.	— 17.0°	Water	54
	145°/.6 mm.	— 34.6°	"	89, 92
	145°/.66 mm.			148
57.5°		— 29.3°	Chloroform	149
	120°/1 mm.	— 31.3°	Water	159
		— 34.6°	"	163
108-10°				193
62.4°		— 15.5°		219
57°				246
58-9°				260

The refractive index is said to be 1.4548 at 20° (89, 92).

(w) *Trimethyl-4-potassium  $\beta$ -methyl glucoside* (164).

(x) 2, 3, 6-Trimethyl glucose reacts only imperfectly (63) with phenyl-carbimide, while the *hydrazone*, *oxime* and the *anilide* are unstable syrups.

(y) *Trimethyl glucose anhydride* < $\alpha$  1, 5> < $\beta$  1, 4>.

Described or mentioned in 194, 207, 210, 214, 216, 231, 238 and 281. This is a colorless oil, stable toward Fehling's, permanganate and bromine (194, 207), contains no hydroxyl group, and is changed by hydrolysis into trimethyl glucose. It produces a crystalline *pyridonium* compound. It is soluble in all solvents, water and petroleic ether. Distillation temperatures are 83-85°/0.1 mm. (207); 84°/0.1 mm., and 80-86°/1 mm. Optical rotation constants are (207):  $-10.1^\circ$  (no solvent);  $-14.6^\circ$  (chloroform); and  $+16.5^\circ$  (water) (all at 16°C.).  $N_D^{15} = 1.4656$  (207). This anhydride is not identical (281) with trimethyl cellulose.

### 2, 3, 6-Triethyl Glucose

Mentioned in 46, 111, 180, 181, 215. The melting point is 100-101°. From triethyl cellulose (215) is secured *triethyl ethyl glucoside* (111), a colorless mobile oil of b.p. 120-123° at 0.2 mm., strongly refractive, and soluble in water and most organic solvents. X-ray diagrams of triethyl glucose have been studied (216).

### Methylated Glucosans

Mentioned in 144, 194, 214, 221, 248 and 241. Trimethyl glucosan is a pale yellow oil (55) giving 2, 3, 5-trimethyl glucose dinitrate (which see) when treated with fuming nitric acid (123). It has a reducing value of 10.6 if glucose is 100 (130). When treated with titanium tetrachloride, a chlorine-containing amorphous substance is secured. The glucosan occurs as an impurity in the trimethyl glucose from the methylated galactosidoglucose of Fischer's (165). Methylated tetraglucosan gives tetramethyl glucose (71, 114, 223), 2, 3, 5-trimethyl glucose (114, 244) and an unidentified dimethyl glucose (114) on hydrolysis. A dimethyl glucosan (241) has already been discussed in a previous paper.

## TETRA-ALKYLATED GLUCOSES

### GENERAL

A large number of papers casually mention tetramethyl glucose without specifically describing the individual sugar under discussion. They are: 4, 5, 9, 10, 14, 15, 16, 26, 28, 34, 37, 41, 43, 52, 59, 60, 63, 65, 68, 73, 78, 88, 94, 104, 111, 113, 118, 120, 139, 147, 148, 152, 155-157, 160, 162, 163, 168, 173, 175, 181, 240, 247, 250, 268, 272, 277, 269, 278. Tetramethyl d-gluconic acid is mentioned in 186, the tetramethyl gluconolactone in 8, 10 and 104, and tetramethyl methyl glucoside in 5, 8, 9, 14, 17, 26, 43, 59, 73, 115, 140, 160, 162, 179 and 246. Mutarotation of tetramethyl glucose is discussed in 26, 90, 122, 134, 151, 190-192 and 197.

It is stated that no tetramethyl glucose is present in the hydrolytic products from trimethyl starch (211), and the same is generally conceded to be true in the case of trimethyl cellulose (60, 61, 83, 92, 207) although one paper reports (150) that 2, 3, 5, 6-tetramethyl glucose is secured in significant amounts and another (18) states that a small amount is obtained. When paullinia tannin is methylated, a tetramethyl glucose, among other things, is obtained from the hydrolytic products (51, 70, 276). The effect of increasing the methoxyl groups on the absorption band has been determined in the case of tetramethyl methyl glucoside (262). Di-ethyl



mercapto-glucose produces a tetramethyl derivative on methylation (153). A 1, 3, 4, 5-tetramethyl lactonic acid is mentioned (243) as well as a tetramethyl beta-ethyl glucoside (264) of boiling point  $118^{\circ}/0.5$  mm. (11). 2, 3, 5, 6-Tetraethyl glucose has not been obtained pure (111).

Derivatives of 2, 3, 4, 5-tetramethyl saccharic acid found mentioned in the literature (67, 176, 232) are: (1) The barium salt, long needles, soluble in water; (2) the silver salt, a white powder; (3) a diamide, composed of colorless glittering rhombic leaflets m.p.  $237^{\circ}$ ,  $[\alpha]_D^{18} + 12.22^{\circ}$ ; (4) a crystalline methyl amide, and (5) the dimethyl ester, of b.p.  $150^{\circ}/1$  mm., crystallizing as white needles or leaflets, m.p.  $68^{\circ}$ . Easily soluble in water, chloroform or ether.  $[\alpha]_D^{18} = +8.88^{\circ}$ . This ester may be prepared directly from saccharic acid (176). The tetramethyl saccharic acid is prepared from 2, 3, 4, 5-tetramethyl gluconic acid,  $[\alpha]_D = +10^{\circ}$ , which, in turn, is obtained from methylated melibionie acid.

A tetramethyl hexose diphosphate may be secured (198) from the calcium salt of candiolin. It is a yellowish oil,  $n_D^{19} = 1.4648$ ,  $[\alpha]_D^{18} = 20.22^{\circ}$  ( $\text{CH}_3\text{OH}$ ) which, when treated with Purdie's reagents, forms the heptamethyl ester, b.p.  $130-140^{\circ}/0.01$  mm., with a rotation of  $20.77^{\circ}$  in chloroform at  $19^{\circ}\text{C}$ .

A 3, 4, 5, 6-tetramethyl glucose is reported which, on reduction, gives 3, 4, 5, 6-tetramethyl sorbitol, which shows optical exaltation in the presence of boric acid (24, 36). 2, 3, 4, 5-Tetramethyl glucose is mentioned in 152 and 246.

### 2, 3, 4, 6-Tetramethyl Glucose

This tetramethyl glucopyranose is without question our most important sugar for the proof of structure. The methyl groups were at first assigned the positions 2, 3, 5 and 6, but the researches of Haworth and co-workers showed that the methyl groups are undoubtedly attached to carbons 2, 3, 4 and 6. There is a definite 2, 3, 5, 6-tetramethyl glucose, of the furanose type, which will be described next.

See references 11, 23, 24, 29, 40, 46, 107, 152, 156, 157, 182-184, 206, 230, 243, 287 and 291 for general mention of this compound, and 166 for a discussion of its reducing power. Its behavior in boric acid solutions under different conditions may be found in 43, 218, 245 and 246. A common methylene-diol is shared with tetramethyl mannose in aqueous solutions (208, 228). The compound mutarotates even in the fused state (203), and does not oxidize to a tetramethyl saccharic acid (223) in various alkaline oxidizing solutions (246). On the other hand, it yields (203, 205) formic acid when it is treated with alkaline hydrogen peroxide solution and also a five-carbon acid. It is further stated to give (117, 173) a 2, 5-dimethyl  $\alpha$ -arabonolactone, methyl esters of a methylated tetric acid and demethylated hexonic and pentonic acids. Since the tetramethyl glucose occurs in hydrolytic products quite frequently along with 2, 3, 6-trimethyl glucose, it is fortunate that the former can be separated (63) by extraction with boiling light petroleum from the latter.

The tetramethyl glucose is not affected by the Grignard reagent (13), by fuming  $\text{HCl}$  (80), forms no osazone (146, 148) and is not digested by organisms (270). The sugar has a bitter taste, is neutral to litmus, readily soluble in organic solvents (2), but sparingly so in ligroin. The crystal form is needle-like (105, 282, 285, 290). It is stated (203) that when the

tetramethyl glucose melting at  $98^{\circ}$  is crystallized rapidly from pure dry ether and ligroin (b.p.  $30-40^{\circ}$ ) the melting point goes up to  $103-104^{\circ}$ . This compound then shows  $[\alpha]_{5461}^{44}=142.3^{\circ}$ , unchanged for six hours. Muta-rotation then proceeded rapidly, and was complete in 198 hours. The refractive index is 1.4583. The compound decomposes with titanium tetrachloride in chloroform, and the investigators (254) were not able to recognize any product in the reaction mixture. It causes no alleviation of the symptoms caused by insulin in mice (91).

The 2, 3, 4, 6-tetramethyl glucose may be prepared from the following methylated substances:

- (a) Alpha- and beta-methyl glucosides (2, 3, 19, 22, 24, 31, 39, 209, 232, 233).
- (b) Tetramethyl methyl d-glucosides (39, 87, 146, 242).
- (c) Glycerol glucoside (109, 110).
- (d) Methylated cellobioside (45, 48, 82, 108, 178).
- (e) Glucose oxime (11, 289).
- (f) Methylated gentiobiose (105, 108).
- (g) Methylated maltose (33).
- (h) Hexobiose of amygdalin (57, 58, 85, 93, 108, 147).
- (i) Methylated arbutin (87).
- (j) Octamethyl sucrose (27, 36, 69, 81, 82, 158).
- (k) 2, 3, 6-Trimethyl beta-methyl glucoside (60, 61, 101, 159, 194, 223, 241).
- (l) Hendecamethyl melezitose (185).
- (m) Hexamethyl amylobiose (162).
- (n) Hexamethyl glucoarabinose (35).
- (o) Pentamethyl aesculin (288).
- (p) Heptamethyl-4- $\beta$ -glucosido- $\alpha$ -methyl mannoside (282).
- (q) Methylated cellotriose (284).
- (r) Trimethyl glucal (285).
- (s) Trimethyl glycogen (290).
- (t) Octamethyl maltobionic acid (290).
- (u) Tetramethyl indican (69).
- (v) Methylated trehalose (125, see also Bredereck, Ber., 63:959 (1930)).
- (w) Polymerized glucosan (71, 114, 223).

The principal physical characteristics are recorded below, the rotation in every case being determined in water.

B. P.	MM.	M. P.	$[\alpha]_D$	Reference
182.5°		81.3°	+ 80.8°	2
		86.8°		7
		88.9°		12
		84°		19, 22, 27, 45, 58
			83.0°	33, 126, 146
		85.7°		35
150.5°		82°		105
182.5°	20	88.9°	83.3°	108, 232
		86.7°	90° → 84.1°	140
		81.3°		159
			83.3°	160
		86°		174
		93.4°	99.9° → 83°	178
		81.2°	82.3°	194
		98.0°		203
			83.7°	208
		88.0°	82.0°	228
		82.4°	79.0°	246
153.6°	0.2	85°		256
		86°	83°	282
		86.8°	84°	284
		84.5°	100° → 83°	285
110°	0.5	94°	91° → 83.2°	288
		86°	91° → 83.2°	290

The rotations in alcohol may be found in 2, 35, 159; in acetone in reference 203; benzene is given in 125; and for the constants in unusual solvents refer to 9.

Derivatives of this tetramethyl glucose are:

(a) *Tetramethyl gluconic acid* (155, 160, 184).

May be prepared from the parent sugar (2, 72, 145, 146, 174, 232, 243). Its *sodium salt* is not digested by organisms (270), the *calcium salt* is a gum, and the *cadmium salt* is a glass (2). Rotations may be found in the references 116, 154 and 170.

(b) *Tetramethyl d-gluconolactone* (76, 140, 184).

It is prepared from (a) (2, 72, 145, 159, 174, 232, 242), from the tetramethyl glucose (99, 109, 116, 255), or by epimerization of tetramethyl delta-mannolactone (188, 257). The rate of hydrolysis of this lactone has been studied (209, 233, 264-265). It is a pale yellow oil, b.p. 106-107°/0.04 mm. (174), with a refractive index of 1.4566 at 14° or 1.4556 at 18°C. (286). Rotations in water are given as: + 98° (170, 264, 265); + 99° → 30.8° (8 hrs.) (174); 101.1° → 25.6° (131); 97.7° → 37.1° (286). The lactone forms a crystalline *amino-lactone*, which is apparently an internal urethane (72, 99). On epimerization, tetramethyl  $\delta$ -mannolactone is formed (189, 232, 235). Oxidation with nitric acid leads to xylotrimethoxy-glutaric acid (137, 145, 159, 160, 176, 209, 232, 233, 242, 255), but it is said (145) that d-dimethoxysuccinic acid is formed, too.

(c) *Tetramethyl d-phenylhydrazide* (2, 13, 131, 174).

Melting point 109-112° (131); 115° (174, 235, 236). Optical rotation is +42.1° (alc.) (174); +50.8° (alc.) (236). Soluble in alcohol, ether and benzene (2).

(d) *Tetramethyl l-gluconolactone* (232).

(e) *Tetramethyl l-phenylhydrazide* (232, 236).

M.p. is 115°;  $[\alpha]_D - 50^\circ$ .

(f) *Tetramethyl oxime* (12, 13).

(g) *Tetramethyl oxime methyl ether* (13).

(h) *Tetramethyl gluconoamide* (72, 99, 286).

Consist of waxy needles, m.p. 68-70°, with  $[\alpha]_D + 77.5^\circ$  in benzene and +60.4° in acetone. Is an aminolactone, and not a true acid amide.

(i) *Tetramethyl gluconocarbimide*.

(j) *Octamethyl diglucose anhydride* (7, 125).

Produced when the tetramethyl glucose is heated in benzene with HCl. It is a syrup, distillable without decomposition, probably a disaccharide of the trehalose types, with a rotation of +135.9° in methyl alcohol.

(k) *Tetramethyl glucose anilide* (12, 13, 21, 162, 168, 188, 189, 285, 289).

The needles possess a melting point of 135° (204); 136-137° (71); 138° (203, 228) and a rotation of +230° → +59° (203) and +226° (methyl alc.) (228). There is also reported the *p-bromanilide*, *o-bromanilide*, *p-toluidide* and the *p-anisidide* (204).

(l) *Tetramethyl beta-methyl glucoside* (9, 43, 45, 69, 81, 87, 101, 164, 194, 217, 241, 249, 264, 265, 286, 288).

This glucoside is not digestible to micro-organisms (132, 270). The colorless oil distils at 108-110°/0.23 mm. (31) or 107-108° at 1 mm. (159). The refractive index is 1.4455 (31). It is said to have a melting point of 42-43° (2); 39-41° (31). The glucoside is readily hydrolyzed by emulsin (3). The following rotations are recorded: -15.8° (alc.) (159); -17° (water) (54, 146); -13.3° (alc.) (31), and -11.6° (3).

(m) *Tetramethyl alpha-methyl glucoside* (9, 69, 264, 265).

Usually prepared directly from  $\alpha$ -methyl glucoside (2, 3, 19, 22). It is not affected by emulsin (3). The syrup is colorless, neutral, with a burning taste, soluble in alcohol and acetone. No action takes place on Fehling's. The boiling point is given at 108°/0.1 mm. (19, 22); 144-152°/20 mm., 75-85°/0.01 mm. (284). Other physical constants are  $n_D = 1.4454$  (19, 22);  $n_D^{14} = 1.4462$  (284);  $d_4^{20} = 1.1082$  (19, 22). Specific rotation in water is given as +147.4° (3, 6), and in alcohol as +153.9° (6) and +154° (54).

(n) *1-Chloro-2, 3, 4, 6-Tetramethyl glucose* (13, 259, 260).



Together with 1, 2, 3, 6-tetramethyl glucose, crystalline octamethyl cellobiose may be synthesized from this compound.

*2, 3, 5, 6-Tetramethyl Glucose*

Discussed in 1, 23, 81, 95, 120, 130, 152, 154, 160, 162, 163, 168, 182, 183. It may be prepared from the following sources:

- (a) Gamma-methyl glucoside (24, 160, 222, 232).
- (b) Methylated lactose (82, 178, 222).
- (c) Methylated maltobionate (176).
- (d) Methylated celloside (47, 49).
- (e) Acetone glucose (160).
- (f) 2, 3, 6-Trimethyl gamma-methyl glucoside (160, 241).
- (g) 3-Monomethyl glucose (228).

This furanose type of tetramethyl glucose reduces alkaline permanganate very readily (29, 160). Conversion into the glucoside takes place very rapidly (29). It distils at 120°/0.03 mm. (249); 100-110°/0.1 mm. (226). For water  $[\alpha]_D = -11.1^\circ$ ;  $-7.2^\circ$ ;  $-9.7^\circ$  (160);  $-7.28^\circ$  (249);  $-27.5^\circ$  (226). In benzene at 20°, the rotation is  $-17.1^\circ$  (187). The refractive index is 1.4525; 1.4585; 1.4563 (160); 1.4450 (226). A number of investigators (47, 49 and 228) give its melting point as 90-94°.

From this sugar have been prepared:

- (a) *Tetramethyl glucose anilide* (24, 228).

Melting point is 135° (228).

- (b) *Tetramethyl glucose acetoacetic acid* (199, 200).

Not obtained pure.

- (c) *Tetramethyl glucose cycloacetic acid and ester* (171, 199, 200).

This does not reduce alkaline copper solutions but instantly decolorizes aqueous permanganate in the cold. See the references for other characteristics.

- (d) *Tetramethyl hexitol* (24).

This is presumed to be a tetramethyl sorbitol.

- (e) *Tetramethyl gluconoamide*.

Long needles, m.p. 91°;  $[\alpha]_D^{24} = +39.2^\circ$  (water).

- (f) *Tetramethyl gluconic acid*.

One worker (24) states that a *pentitol ether* is obtained, in addition, when the parent tetramethyl glucose is oxidized. The acid may be prepared from the tetramethyl glucose (24, 76, 133, 170, 176, 232, 237) or from methylated maltobionic acid (33, 202, 232, 290). The acid has  $[\alpha]_D = +27.7^\circ$  (160, 170, 176).

- (g) *Tetramethyl glucose phenylhydrazide* (33, 131, 137, 177, 178, 202, 228, 232, 190).

It is claimed to be a resinous product (24), but the following melting points have been recorded: 131-134° (131); 134-136° (232, 290); 135-136° (178, 235); 136° (228).

- (h) *Tetramethyl gamma-gluconolactone*.

Methods of preparation include: (1) 2, 3, 6-trimethyl gamma-gluconolactone (131, 137, 174, 232); (2) methylated lactobionate (177); (3) epimerization of tetramethyl gamma-mannolactone (232, 235) or (4) from the gluconic acid (f) (24, 176, 233). The rate of hydrolysis has been studied (209, 233, 264, 265). On degradation d-dimethyl tartaric acid (209, 232, 233) or d-methoxysuccinic acid (identified by the crystalline amide and methyl amide) is formed (145, 174, 176, 237). The melting point varied between 25.5-27° (170, 178, 228, 264, 265, 290). It is more stable (174) than the delta-lactone. Properties of the substance as a syrup: b. p. 115-120°/0.04 mm.;  $n_D^{15.3} = 1.4486$ ,  $n_D^{14} = 1.4501$  (170);  $n^{15} = 1.4490$  (290). Aqueous rotations are:  $[\alpha]_D^{18} = +61.0^\circ$  (30 mins.)  $\rightarrow +58.2^\circ$  (1 day) (290);  $+57^\circ$  (2 days) (290);  $[\alpha]_D^{12} = 62.5^\circ \rightarrow 32.9^\circ$  (170);  $61.5^\circ \rightarrow 39.6^\circ$  (131);  $62^\circ$  (264, 265).

- (i) *Tetramethyl alpha-methyl glucoside* (24, 54, 187, 228, 241, 249, 264, 265).

A colorless syrup,  $n_D^{16} = 1.4440$ ;  $[\alpha]_D^{16} = -20.4^\circ$  (water);  $[\alpha]_D^{24} = -24.15^\circ$  (methyl alc.) (1, 154, 187, 286).

- (j) *Tetramethyl beta-methyl glucoside* (1, 24, 54, 154, 187, 217, 241).

Does not relieve symptoms caused by insulin in mice (91). Melts at 40-41° (264, 265); distils at 142-144° (226); 107-110°/0.2 mm. (249); 115-116°/0.5 mm. (24). It is hydrolyzed slowly by N/100 HCl at 40° and rapidly at 100°. The other physical constants are recorded as follows:  $n_D^{20} = 1.4472$  (226);  $[\alpha]_D^{17} = +18.38^\circ$  (CH<sub>3</sub>OH);  $-17.65^\circ$  (96% alc.) (222);  $[\alpha]_D^{20} = -17^\circ$  (226);  $-17.75^\circ$  (final—CH<sub>3</sub>OH) (187); and  $-9.2^\circ$  in 5 per cent solution in N/100 HCl (24);  $-14.6^\circ$  (water) (249).

#### PENTAMETHYL GLUCOSE

This undoubtedly occurs as the aldehyde form (262) with the methyl groups attached to carbons 2, 3, 4, 5 and 6. It gives the characteristic carbonyl absorption band. It is mentioned in 228, 253, 258 and 289 without any detailed description. Calcium gluconate is the usual basis for its preparation (116, 139), but it may also be prepared from the diethyl mercapto compound (153). Directions for the preparation of *pentamethyl diethyl mercapto-glucose* may be found in 153, 274 and 275. This mercapto-compound has a boiling point of 145-155° at 0.6 mm.,  $n_D^{20} = 1.4884$ , and  $d_4^{20} = 1.0834$ . The rotation in methyl alcohol is 19.2°. It does not reduce Fehling's and has a garlic odor.

2, 3, 4, 5, 6-Pentamethyl glucose distils at 108-110°/0.4 mm.,  $n_D^{20} = 1.4467$ ;  $d_4^{20} = 1.0944$ ;  $[\alpha]_D = -11.9^\circ$  (18 hrs.) (153). Other rotations found in the literature are  $-35.4^\circ$  at 20°C. (186) and  $+53.7^\circ$  in a solu-

tion of NaOH (116, 139), which falls to  $+22.5^{\circ}$  when the solution is neutralized. It reduces Fehling's only on boiling, but ammoniacal silver nitrate is reduced at room temperature. Permanganate is reduced readily. The sugar forms a *dimethyl acetal* (139, 153, 186), which distils at  $95^{\circ}/0.86$  mm.,  $n_D^{20} = 1.4373$  and rotates  $+15.09^{\circ}$  in methyl alcohol.

The pentamethyl glucose is not fermentable by a large group of organisms (270). The rotation dispersion data has been calculated (227) as well as the reducing power (166). Glucose oxime, on methylation, does not give the open chain pentamethyl glucose as would be expected (12, 289).

## REFERENCES

- 1903
  1. Purdie, T., and R. C. Bridgett—J. Chem. Soc., (London) **83**:1037-1041.
  2. Purdie, T., and J. C. Irvine—J. Chem. Soc., (London) **83**:1021-1037.
- 1904
  3. Purdie, T., and J. C. Irvine—J. Chem. Soc., (London) **85**:1049-1070.
- 1905
  4. Fenton, H. J. H.—Ann. Reports Prog. Chem., **2**:82.
  5. Irvine, J. C., and A. Cameron—J. Chem. Soc., (London) **87**:900-909.
  6. Irvine, J. C., and A. M. Moodie—J. Chem. Soc., (London) **87**:1462-1468.
  7. Purdie, T., and J. C. Irvine—J. Chem. Soc., (London) **87**:1022-1030.
- 1906
  8. Fenton, H. J. H.—Ann. Reports Prog. Chem., **3**:89.
  9. Irvine, J. C., and A. M. Moodie—J. Chem. Soc., (London) **89**:1578-1590.
  10. Irvine, J. C., and R. E. Rose—J. Chem. Soc., (London) **89**:814-822.
- 1908
  11. Desch, C. H., and G. T. Morgan—Ann. Reports Prog. Chem., **5**:87.
  12. Irvine, J. C., and R. Gilmour—J. Chem. Soc., (London) **93**:1429-1441.
  13. Irvine, J. C., and A. M. Moodie—J. Chem. Soc., (London) **93**:95-107.
- 1912
  14. Le Sueur, H. R.—Ann. Reports Prog. Chem., **9**:94-100.
- 1913
  15. Irvine, J. C., and J. P. Scott—J. Chem. Soc., (London) **103**:564-575.
  16. Irvine, J. C., and J. P. Scott—J. Chem. Soc., (London) **103**:575-586.
  17. Irvine, J. C., R. F. Thomson and C. S. Garrett—J. Chem. Soc., (London) **103**:238-249.
- 1914
  18. Denham, W. S., and H. Woodhouse—J. Chem. Soc., (London) **105**:2357-2368.
  19. Haworth, W. N.—Proc. Chem. Soc., (London) **30**:293-294.
  20. Irvine, J. C.—Ann. Reports Prog. Chem. **11**:84.
  21. Irvine, J. C., and B. M. Paterson—J. Chem. Soc., (London) **105**:915-923.
- 1915
  22. Haworth, W. N.—J. Chem. Soc., (London) **107**:8-16.
  23. Irvine, J. C.—Ann. Reports Prog. Chem., (London) **12**:70.
  24. Irvine, J. C., A. W. Fyfe and T. P. Hogg—J. Chem. Soc., (London) **107**:524-541.
  25. Irvine, J. C., and J. L. A. MacDonald—J. Chem. Soc., (London) **107**:1701-1710.
  26. Irvine, J. C., and E. S. Steele—J. Chem. Soc., (London) **107**:1230-1240.
- 1916
  27. Haworth, W. N., and J. Law—J. Chem. Soc., (London) **109**:1314-1325.
  28. Irvine, J. C.—Ann. Reports Prog. Chem., (London) **13**:83.
- 1917
  29. Denham, W. S., and H. Woodhouse—J. Chem. Soc., (London) **111**:244-249.
  30. Irvine, J. O.—Ann. Reports Prog. Chem., **14**:75-82.

## 1918

31. Haworth, W. N., and G. C. Leitch—*J. Chem. Soc.*, (London) 113:118-199.  
32. Irvine, J. C.—*Ann. Reports Prog. Chem.*, (London) 15:63-69.

## 1919

33. Haworth, W. N., and G. C. Leitch—*J. Chem. Soc.*, (London) 115:809-817.  
34. Irvine, J. C.—*Ann. Reports Prog. Chem.*, (London) 16:75-84.  
35. Irvine, J. C., and J. S. Dick—*J. Chem. Soc.*, (London) 115:593-602.

## 1920

36. Haworth, W. N.—*J. Chem. Soc.*, (London) 117:199-208.  
37. Hess, K., and W. Wittelsbach—*Zeits. Elektrochem.*, 26:232-251.  
38. Irvine, J. C., and C. W. Soutar—*J. Chem. Soc.*, (London) 117:1489-1500.  
39. Irvine, J. C., and E. S. Steele—*J. Chem. Soc.*, (London) 117:1474-1489.  
40. Karrer, P.—*Helv. Chim. Acta* 3:258-260.  
41. Pickard, R. H.—*Ann. Reports Prog. Chem.*, (London) 17:64.  
42. Pictet, A., and M. Cramer—*Helv. Chim. Acta*, 3:640-644.

## 1921

43. Boeseken, J., and H. Couvert—*Rec. trav. chim.*, 40:354-380; *Akad. Wetenschappen Amsterdam*, 29:924-934 (1920).  
44. Denham, W. S.—*J. Chem. Soc.*, (London) 119:77-81.  
45. Haworth, W. N., and E. L. Hirst—*J. Chem. Soc.*, (London) 119:193-201.  
46. Hess, K., W. Wittelsbach and E. Messmer—*Zeits. angew. Chem.*, 34:449-454.  
47. Karrer, P.—*Naturwissenschaften*, 9:399-403.  
48. Karrer, P., and F. Widmer—*Helv. Chim. Acta*, 4:295-297.  
49. Karrer, P., and W. Widmer—*Helv. Chim. Acta*, 4:174-184.  
50. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, 48:233-248.  
51. Nierenstein, M., C. W. Spiers and A. Geake—*J. Chem. Soc.*, (London) 119:275-286  
52. Pickard, R. H.—*Ann. Reports Prog. Chem.*, (London) 18:73-76.  
53. Reilly, J.—*Helv. Chim. Acta*, 4:616-621.

## 1922

54. Carruthers, A., and E. L. Hirst—*J. Chem. Soc.*, (London) 121:2299-2308.  
55. Cramer, M., and E. H. Cox—*Helv. Chim. Acta*, 5:884.  
56. Freudenberg, K., and O. Ivers—*Ber.*, 55:929-941.  
57. Haworth, W. N.—*Ann. Reports Prog. Chem.*, (London) 19:77.  
58. Haworth, W. N., and G. C. Leitch—*J. Chem. Soc.*, (London) 121:1921-1929.  
59. Irvine, J. C., and co-workers—*Brit. Ass'n Reports, Chem News*, 125:165-170; 181-186.  
60. Irvine, J. C., and co-workers—*J. Soc. Chem. Ind.*, 41:362-365R.  
61. Irvine, J. C.—*Report Brit. Ass'n Adv. Sci.*, 90:33-48.  
62. Irvine, J. C., W. S. Denham and E. L. Hirst—*Chem. Trade J.*, (London) 79:291.  
63. Irvine, J. C., and E. L. Hirst—*J. Chem. Soc.*, (London) 121:1213-1223.  
64. Irvine, J. C., and J. Patterson—*J. Chem. Soc.*, (London) 121:2146-2161.  
65. Irvine, J. C., E. S. Steele and M. I. Shannon—*J. Chem. Soc.*, (London) 121:1060-1078.  
66. Karrer, P.—*Zeits. angew. Chem.*, 35:85-90.  
67. Karrer, P., and J. Peyer—*Helv. Chim. Acta*, 5:577-581.  
68. Karrer, P., and A. P. Smirnoff—*Helv. Chim. Acta*, 5:187-201.  
69. Macbeth, A. K., and J. Pryde—*J. Chem. Soc.*, (London) 121:1660-1668.  
70. Nierenstein, M.—*J. Chem. Soc.*, (London) 121:23-28.  
71. Pringsheim, H., and K. Schmalz—*Ber.*, 55:3001-3007.  
72. Pryde, J.—*J. Soc. Chem. Ind.*, 41:365R.

## 1923

73. Bridel, M.—*J. Chem. Soc.*, (London) 123:1277-1279.  
74. Haworth, W. N., E. L. Hirst and D. A. Ruell—*J. Chem. Soc.*, (London) 123:3125-3131.  
75. Haworth, W. N., E. L. Hirst and D. A. Ruell—*J. Soc. Chem. Ind.*, 43:1139.  
76. Haworth, W. N., and W. H. Linnell—*J. Chem. Soc.*, (London) 123:294-301.  
77. Haworth, W. N., and J. G. Mitchell—*J. Chem. Soc.*, (London) 123:301-310.  
78. Haworth, W. N., and B. Wylam—*J. Soc. Chem. Ind.*, 43:1139.  
79. Hess, K., W. Weltzien and E. Messmer—*Annalen*, 435:1-144.



80. Hirst, E. L., and D. R. Morrison—*J. Chem. Soc., (London)* 123:3226-3235.
81. Irvine, J. C.—*J. Ind. and Eng. Chem.,* 15:1162-1164.
82. Irvine, J. C.—*J. Chem. Soc., (London)* 123:898-921.
83. Irvine, J. C., and E. L. Hirst—*J. Chem. Soc., (London)* 123:518-532.
84. Komatsu, S., T. Inoue and R. Nakai—*Mem. Coll. Sci. Kyoto Imper. Univ.,* 7:25-30.
85. Kuhn, R.—*Ber.,* 56:857-862.
86. Ling, A. R., and D. R. Nanji—*J. Chem. Soc., (London)* 123:2666-2688.
87. Macbeth, A. K., and T. Mackay—*J. Chem. Soc., (London)* 123:717-724.
88. Pryde, J.—*J. Chem. Soc., (London)* 123:1808-1815.
89. Schlubach, H. H., and K. Moog—*Ber.,* 56:1957-1963.

## 1924

90. Baker, J. W., J. F. Ingold and C. K. Thorpe—*J. Chem. Soc., (London)* 125:268-291.
91. Herring, P. T., J. C. Irvine and J. J. R. MacLeod—*Biochem. J.,* 18:1023-1042.
92. Hess, K.—*Z. angew. Chem.,* 37:993.
93. Hudson, C. S.—*J. Amer. Chem. Soc.,* 46:483-489.
94. Irvine, J. C.—*Chem. Reviews,* 1:41-71.
95. Irvine, J. C., and W. Burt—*J. Chem. Soc., (London)* 125:1343-1348.
96. Irvine, J. C., and H. S. Gilchrist—*J. Chem. Soc., (London)* 125:1-10.
97. Irvine, J. C., and E. L. Hirst—*J. Chem. Soc., (London)* 125:15-25.
98. Irvine, J. C., H. Pringsheim and J. MacDonald—*J. Chem. Soc., (London)* 125:942-947.
99. Irvine, J. C., and J. Pryde—*J. Chem. Soc., (London)* 125:1045-1049.
100. Karrer, P., and K. Nishida—*Helv. Chim. Acta,* 7:363-370.
101. Macbeth, A. K., and J. Mackay—*J. Chem. Soc., (London)* 125:1513-1521.
102. Ohle, H.—*Ber.,* 57:403-409.
103. Pacsu, E.—*Ber.,* 57:849-853.
104. Pryde, J.—*J. Chem. Soc., (London)* 125:520-522.
105. Zemplén, G.—*Ber.,* 57:698-704.
106. Zwicker, J. J. L.—*Chem. Weekblad,* 21:349-352.

## 1925

107. Baker, S., and W. N. Haworth—*J. Chem. Soc., (London)* 127:365-369.
108. Bridel, M.—*J. Pharm. et de Chimie,* 1:116-122; 161-166; 215-225; 253-261.
109. Doree, C.—*Ann. Reports Prog. Chem., (London)* 22:82-103.
110. Gilchrist, H. L., and C. B. Purves—*J. Chem. Soc., (London)* 127:2735-2745.
111. Hess, K., and G. Salzmänn—*Annalen,* 445:111-122.
112. Hess, K., and W. Weltzien—*Annalen,* 442:46-60.
113. Irvine, J. C., and J. W. H. Oldham—*J. Chem. Soc., (London)* 127:2729-2735; *Proc. Chem. Soc., (London)* 41:113.
114. Irvine, J. C., and J. W. H. Oldham—*J. Chem. Soc., (London)* 127:2903-2922.
115. Kuhn, R., and H. H. Schlubach—*Zeits. physiol. Chem.,* 143:154-157.
116. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.,* 65:535-544.
117. Lewis, W. L., and E. L. Gustus—*Abst. Org. Div., A. C. S. Baltimore.*
118. Lewis, W. L., M. L. Wolfson and R. D. Greene—*Abst. Org. Div., A. C. S., Baltimore.*
119. Ling, A. R., and D. R. Nanji—*J. Chem. Soc., (London)* 127:629-636.
120. Lowry, T. M.—*J. Chem. Soc., (London)* 127:1371-1385.
121. Lowry, T. M., and J. Faulkner—*J. Chem. Soc., (London)* 127:2883-2887.
122. Lowry, T. M., and E. M. Richards—*J. Chem. Soc., (London)* 127:1385-1401.
123. Oldham, J. W. H.—*J. Chem. Soc., (London)* 127:2840-2845.
124. Pringsheim, H., W. Knoll and E. Kasten—*Ber.,* 58:2135-2143.
125. Schlubach, H. H., and K. Maurer—*Ber.,* 58:1178-1184.
126. Schlubach, H. H., and G. Rauchsches—*Ber.,* 58:1842-1850.
127. Schlubach, H. H., and W. Rauchenberger—*Ber.,* 58:1184-1189.
128. Weltzien, W., and R. Singer—*Annalen,* 443:71-112.
129. Whittier, E. O.—*Chem. Reviews,* 2:85-125.
130. Zemplén, G., and G. Braun—*Ber.,* 58:2566-2570.

## 1926

131. Charlton, W., W. N. Haworth and S. Peat—*J. Chem. Soc., (London)* 129:89-101.
132. Coles, H. W.—*Plant Physiology,* 1:379-385.
133. Cooper, C. J. A., W. N. Haworth and S. Peat—*J. Chem. Soc., (London)* 129:876-880.

134. Faulkner, I. J., and T. M. Lowry—*J. Chem. Soc.*, (London) **129**:1938-1943.
135. Fear, C. M., and R. C. Menzies—*J. Chem. Soc.*, (London) **129**:937-940.
136. Gray, Harry LeB.—*J. Ind. and Eng. Chem.*, **18**:811.
137. Haworth, W. N.—*Ann. Reports Prog. Chem.*, (London) **23**:74-97.
138. Haworth, W. N., and E. L. Hirst—*J. Chem. Soc.*, (London) **129**:1858-1868.
139. Haworth, W. N., and V. S. Nicholson—*J. Chem. Soc.*, (London) **129**:1899-1902.
140. Haworth, W. N., and W. G. Sedgwick—*J. Chem. Soc.*, (London) **129**:2573-2580.
141. Hees, H., and C. Tropp—*Centr. Bakt. Parasitenk.*, I Abt. **100**:273-284.
142. Helferich, B., W. Klein and W. Schafer—*Ber.*, **59**:79-85.
143. Hess, K., and H. Friese—*Annalen*, **450**:40-58.
144. Hess, K., and H. Piehlmayr—*Annalen*, **450**:29-40.
145. Hirst, E. L.—*J. Chem. Soc.*, (London) **129**:350-357.
146. Hudson, C. S.—*J. Amer. Chem. Soc.*, **48**:1434-1443.
147. Hudson, C. S.—*Scientific Papers of the Bur. of Standards*, No. 533.
148. Irvine, J. C., and I. M. Black—*J. Chem. Soc.*, (London) **129**:862-875.
149. Irvine, J. C., and J. MacDonald—*J. Chem. Soc.*, (London) **129**:1502-1518.
150. Irvine, J. C., and G. J. Robertson—*J. Chem. Soc.*, (London) **129**:1488-1501.
151. Jones, G., and T. M. Lowry—*J. Chem. Soc.*, (London) **129**:720-723.
152. Leibowitz, J.—*Zeits. angew. Chem.*, **39**:1143-1148; 1240-1249.
153. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, **69**:175-180.
154. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, **70**:343-353.
155. Levene, P. A., and H. S. Simms—*J. Biol. Chem.*, **68**:737-749.
156. Lewis, W. L., and R. D. Greene—*Science*, **64**:206.
157. Lewis, W. L., and M. L. Wolfrom—*Abst. Org. Div.*, A. C. S., Tulsa, Okla.
158. McOwan, G.—*J. Chem. Soc.*, (London) **129**:1937-1946.
159. Micheel, F., and K. Hess—*Annalen*, **449**:146-155.
160. Micheel, F., and K. Hess—*Annalen*, **450**:21-29.
161. Pringsheim, H.—*Naturwissenschaften*, **14**:198.
162. Pringsheim, H., and A. Steingroever—*Ber.*, **59**:1001-1006.
163. Schlubach, H. H., and H. v. Bomhard—*Ber.*, **59**:845-848.
164. Schlubach, H. H., and H. Firgau—*Ber.*, **59**:2100-2102.
165. Schlubach, H. H., and W. Rauchenberger—*Ber.*, **59**:2102-2106.
166. Sobotka, H.—*J. Biol. Chem.*, **69**:267-275.
167. Zemplen, G.—*Ber.*, **59**:2402-2413.
168. Zemplen, G., and G. Braun—*Ber.*, **59**:2230-2241.

## 1927

169. Charlton, W., W. N. Haworth and W. J. Hickenbottom—*J. Chem. Soc.*, (London) 1527-1536.
170. Drew, H. D. K., E. H. Goodyear and W. N. Haworth—*J. Chem. Soc.*, (London) 1237-1245.
171. Eaton, E. P., and E. S. West—*J. Biol. Chem.*, **75**:283-288.
172. Gray, H. LeB., and C. J. Staub—*Chem. Reviews*, **4**:355-373.
173. Gustus, E. L., and W. L. Lewis—*J. Amer. Chem. Soc.*, **49**:1512-1521.
174. Haworth, W. N., E. L. Hirst and E. J. Miller—*J. Chem. Soc.*, (London) 2436-2443.
175. Haworth, W. N., E. L. Hirst and V. S. Nicholson—*J. Chem. Soc.*, (London) 1513-1526.
176. Haworth, W. N., J. V. Loach and C. W. Long—*J. Chem. Soc.*, (London) 3146-3155.
177. Haworth, W. N., and C. W. Long—*J. Chem. Soc.*, (London) 544-548.
178. Haworth, W. N., C. W. Long and J. H. G. Plant—*J. Chem. Soc.*, (London) 2809-2814.
179. Helferich, B., and A. Schneidmuller—*Ber.*, **60**:2002-2005.
180. Hess, K., and A. Müller—*Annalen*, **455**:205-214.
181. Hess, K., W. Wittelsbach and E. Messmer—*Zeits. angew. Chem.*, **34**:449-454.
182. Irvine, J. C.—*Chem. Reviews*, **4**:203-229.
183. Irvine, J. C.—*Carbohydrates*. Columbia Univ. Press, N. Y.
184. Josephson, K.—*Svensk. Kem. Tids.*, **39**:36-55.
185. Leitch, G. C.—*J. Chem. Soc.*, (London) 588-594.
186. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, **74**:695-699.
187. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, **74**:701-711.
188. Lewis, W. L., and R. D. Greene—*Detroit meeting*, A. C. S.
189. Lewis, W. L., and M. L. Wolfrom—*Detroit meeting*, A. C. S.
190. Lowry, T. M.—*Zeits. Physik. Chem.*, **130**:125-145.
191. Lowry, T. M.—*Chem. Reviews*, **4**:231-253.
192. Lowry, T. M.—*J. Chem. Soc.*, (London) 2554-2565.

193. Micheel, F.—*Annalen*, **456**:69-86.
194. Micheel, F., and K. Hess—*Ber.*, **60**:1898-1906.
195. Ohle, H.—*Ber.*, **60**:1168-1174.
196. Pryde, J., and R. W. Humphreys—*J. Chem. Soc.*, (London) 559-565.
197. Richards, E. M., I. J. Faulkner and T. M. Lowry—*J. Chem. Soc.*, (London) 1733-1739.
198. Schlubach, H. H., and W. Rauchenberger—*Ber.*, **60**:1178-1179.
199. West, E. S.—*J. Biol. Chem.*, **74**:xlii.
200. West, E. S.—*J. Biol. Chem.*, **74**:561-589.
201. Zemplen, G.—*Ber.*, **60**:923-930.
202. Zemplen, G.—*Ber.*, **60**:1555-1564.

## 1928

203. Baker, J. W.—*J. Chem. Soc.*, (London) 1583-1593.
204. Baker, J. W.—*J. Chem. Soc.*, (London) 1979-1987.
205. Evans, W. L., and D. C. O'Donnell—*J. Amer. Chem. Soc.*, **50**:2543-2556.
206. Evans, W. L., W. D. Nicoll, G. C. Strouse and C. E. Waring—*J. Amer. Chem. Soc.*, **50**:2267-2285.
207. Freudenberg, K., and E. Braun—*Annalen*, **460**:288-304.
208. Greene, R. D., and W. L. Lewis—*J. Amer. Chem. Soc.*, **50**:2813-2825.
209. Haworth, W. N.—*Helv. Chim. Acta*, **11**:534-548.
210. Haworth, W. N., and E. L. Hirst—*Ann. Reports Prog. Chem.*, (London) **25**:97-100.
211. Haworth, W. N., E. L. Hirst and J. I. Webb—*J. Chem. Soc.*, (London) 2681-2690.
212. Haworth, W. N., and A. Learner—*J. Chem. Soc.*, (London) 619-625.
213. Helferich, B.—*Zeits. angew. Chem.*, **41**:871-875.
214. Hess, K., and F. Micheel—*Annalen*, **466**:100-114.
215. Hess, K., and A. Müller—*Annalen*, **466**:94-99.
216. Hess, K., and C. Trogus—*Ber.*, **61**:1982-1996.
217. Levene, P. A., and G. M. Meyer—*J. Biol. Chem.*, **76**:513-519.
218. Levy, M., and E. A. Doisy—*J. Biol. Chem.*, **77**:733-751.
219. Linnell, W. H.—*J. Pharm. Soc.*, **1**:200-209.
220. Meyer, K. H.—*Zeits. angew. Chem.*, **41**:935-946.
221. Meyer, K. H., and H. Mark—*Ber.*, **61**:2432-2436.
222. Micheel, F., and O. Littmann—*Annalen*, **466**:115-130.
223. Montonna, R. E.—*Paper Trade J.*, **86**:No. 18, 61-70.
224. Rigby, G. W.—*J. Amer. Chem. Soc.*, **50**:3364-3370.
225. Scheiber, J.—*Farbe u. Lack.*, **334-5**; 346-8.
226. Schlubach, H. H., F. Trefz and W. Rauchenberger—*Ber.*, **61**:2368-2371.
227. Wagner-Jauregg, T.—*Helv. Chim. Acta*, **11**:786-789.
228. Wolfram, M. L., and W. L. Lewis—*J. Amer. Chem. Soc.*, **50**:837-854.
- Anderson, C. G., W. Charlton and W. N. Haworth—*J. Chem. Soc.*, (London) 1329-1337 (1929).

## 1929

229. Coles, H. W., L. D. Goodhue and R. M. Hixon—*J. Amer. Chem. Soc.*, **51**:519-524.
230. Evans, W. L.—*Chem. Reviews*, **6**:281-315.
231. Freudenberg, K.—*Ber.*, **62**:383-386.
232. Haworth, W. N.—*The Constitution of Sugars*. London: Edward Arnold and Co.
233. Haworth, W. N.—*Bull. soc. chim.*, **45**:1-21.
234. Haworth, W. N., E. L. Hirst and J. I. Webb—*J. Chem. Soc.*, (London) 2479-2485.
235. Haworth, W. N., and C. W. Long—*J. Chem. Soc.*, (London) 345-350.
236. Haworth, W. N., and S. Peat—*J. Chem. Soc.*, (London) 350-357.
237. Haworth, W. N., and C. R. Porter—*J. Chem. Soc.*, (London) 2796-2806.
238. Hess, K.—*Ber.*, **62**:924-927.
239. Irvine, J. C.—*Rec. trav. chim.*, **48**:813-816.
240. Irvine, J. C., J. W. H. Oldham and A. F. Skinner—*J. Amer. Chem. Soc.*; **51**:1279-1293.
241. Irvine, J. C., H. Pringsheim and A. F. Skinner—*Ber.*, **62**:2372-2378.
242. Isbell, H. S.—*Bur. Standards J. Res.*, **3**:1041.
243. Josephson, K.—*Svensk. kem. Tids.*, **41**:24-48.
244. Josephson, K.—*Ber.*, **62**:313-316.
245. Levy, M.—*J. Biol. Chem.*, **84**:763-769.
246. Levy, M., and E. A. Doisy—*J. Biol. Chem.*, **84**:749-762.
247. Lowry, T. M.—*J. Phys. Chem.*, **33**:9-21.

248. Meyer, K. H., H. Hopff and H. Mark—Ber., 26:1103-1112.
249. Ohle, H., and L. v. Vargha—Ber., 62:2435-2444.
250. Spoehr, H. A., and H. H. Strain—J. Biol. Chem., 85:365-384.
251. Walton, R. P.—A Comprehensive Survey of Starch Chemistry. Chem. Catalog Co., N. Y.
252. Willstätter, R., and L. Zechmeister—Ber., 62:722-725.
253. Wolf from, M. L.—J. Amer. Chem. Soc., 51:2188-2193.
254. Zemplén, G., and Z. Csürös—Ber., 62:993-996.

## 1930

255. Ambler, J. A.—J. Chem. Ed., 7:1599-1601.
256. Anderson, E., and L. Otis—J. Amer. Chem. Soc., 52:4461-4470.
257. Bott, H. G., W. N. Haworth and E. L. Hirst—J. Chem. Soc., (London) 2653-2659.
258. Brigl, P., and H. Muhlschlegel—Ber., 63:1551-1557.
259. Freudenberg, K.—Naturwissenschaften, 18:393.
260. Freudenberg, K., C. C. Anderson, Y. Go, K. Friedrich and N. W. Richtmyer—Ber., 63:1961-1966.
261. Goodhue, L. D., A. White and R. M. Hixon—J. Amer. Chem. Soc., 52:3191-3195.
262. Goos, F., H. H. Schlubach and G. A. Schroter—Zeits. physiol. Chem., 186:148-156.
263. Gray, H. LeB.—J. Chem. Ed., 7:1803-1811.
264. Haworth, W. N., and E. L. Hirst—J. Chem. Soc., (London) 2615-2635.
265. Haworth, W. N., E. L. Hirst and J. A. B. Smith—J. Chem. Soc., (London) 2659-2663.
266. Hibbert, H., and R. S. Tipson—J. Amer. Chem. Soc., 52:2582.
267. Hudson, C. S.—J. Amer. Chem. Soc., 52:1680-1700.
268. Hudson, C. S.—J. Amer. Chem. Soc., 52:1707-1708.
269. Hudson, C. S.—Org. Symposium, Columbus, January.
270. Kendall, A. I., and C. E. Gross—J. Infect. Dis., 47:249-260.
271. Levene, P. A., and L. A. Mikeska—J. Biol. Chem., 88:791-798.
272. Montgomery, E. M., and C. S. Hudson—J. Amer. Chem. Soc., 52:2101-2106.
273. Nishida, K., and H. Hashima—J. Dep't Agr. Kyushu Imp. Univ., 2:277-360.
274. Papadakis, P. E.—J. Amer. Chem. Soc., 52:2147-2149.
275. Papadakis, P. E.—J. Amer. Chem. Soc., 52:3465.
276. Schmidt, O.—Annalen, 479:1-10.
277. Shaffer, P. A., and T. E. Friedemann—J. Biol. Chem., 86:345-374.
278. Saudinger, H., and O. Schweitzer—Ber., 63:2317-2330.
279. v. Wacek, A.—Ber., 63:282-296.
280. Zemplén, G.—Ber., 63:1820-1823.

## 1931

281. Freudenberg, K.—Indianapolis meeting, A. C. S., 1931.
282. Haworth, W. N., E. L. Hirst and H. R. L. Streight—J. Chem. Soc., (London) 1349-1354.
283. Haworth, W. N., E. L. Hirst and H. A. Thomas—J. Chem. Soc., (London) 821-824.
284. Haworth, W. N., E. L. Hirst and H. A. Thomas—J. Chem. Soc., (London) 825-829.
285. Hirst, E. L., and C. S. Woolvin—J. Chem. Soc., (London) 1131-1137.
286. Humphreys, R. W., J. Pryde and E. T. Waters—J. Chem. Soc., (London) 1298-1304.
287. Levene, P. A., G. M. Meyer and A. L. Raymond—J. Biol. Chem., 31:497-504.
288. Macbeth, A.—J. Chem. Soc., (London) 1288-1290.
289. Wolf from, M. L., and W. R. Brode—J. Amer. Chem. Soc., 53:2279-2281.
- Wolf from, M. L., and A. Thompson—J. Amer. Chem. Soc., 53:622-632.
290. Haworth, W. N., and E. G. V. Percival—J. Chem. Soc., (London) 1342-1349.
291. Gross, C. E., and W. L. Lewis—J. Amer. Chem. Soc., 53:2772-2784.



# STUDIES ON SULFUR OXIDATION<sup>1</sup>

DIONISIO I. AQUINO<sup>2</sup>

*From the Laboratory of Soil Bacteriology, Iowa State College*

Accepted for publication July 20, 1931

The importance of sulfur as one of the elements indispensable for plant growth has been noted by numerous investigators. It has been definitely proved that plants assimilate sulfur in the form of sulfates and recent studies of the sulfur content of plants have shown that certain crops use considerable quantities of the element for their growth.

The fact that many cultivated soils have shown a rather low total sulfur content has led to the use of sulfur as a fertilizing material and interest has been aroused in the effects of sulfur and sulfur compounds on various soil conditions.

The occurrence, numbers and activities of microorganisms in the soil and their relation to the fertility problem, have in recent years been the subject of many investigations. Some very interesting results have been secured from the study of the factors affecting biological activities in the soil. Various soil treatments have been found to exert a marked influence on the numbers of microorganisms, and on the ammonifying, nitrifying, nitrogen fixing, cellulose decomposing, sulfur oxidizing and crop producing powers of the soil.

Although scientists have shown that the oxidation of sulfur to sulfate in the soil ("sulfonation") is brought about chiefly by biological agencies, comparatively little work has dealt with the bacterial activities involved. Therefore, studies of the effect of various fertilizer treatments and of other factors which may influence bacterial activities in the production of greater amounts of sulfates in the soil are of importance. It is also of interest to determine whether or not there is any relation between the power of soils to oxidize sulfur and their ability to produce crops.

The work reported in the following pages was planned to throw some light on the sulfur oxidation and its occurrence under various soil conditions.

## REVIEW OF LITERATURE

### STUDIES ON SULFUR OXIDATION

The fact that sulfur is transformed into sulfates in the soil has long been known (36). More recently extensive studies of sulfur oxidation and its relation to the production of soluble phosphates have been carried on at the New Jersey Agricultural Experiment Station (33, 38).

<sup>1</sup>Part of a dissertation submitted to the Graduate Faculty of Iowa State College in partial fulfillment of the requirements for the degree Doctor of Philosophy.

<sup>2</sup>The author wishes to express his indebtedness to Dr. P. E. Brown for his helpful suggestions in outlining the problem and in reading this manuscript. Thanks are due Dr. F. B. Smith and Dr. R. H. Walker for timely advice and encouragement during the progress of the work.

Simon and Schollenberger (49) and Stephenson (51) found that the rate of sulfur oxidation was dependent on the degree of fineness of the fertilizing material. This result agreed with the finding of Kappen and Quensell (27).

Martin (37) recognized that environmental conditions play an important role in the oxidation of sulfur in the soil. Lipman (29) emphasized temperature, and Brown and Kellogg (11) showed that soil composition, treatment, texture, moisture content, temperature and aeration are all important factors.

Brown and Gwinn (8) reported that the transformation of sulfur to sulfate was more readily accomplished in the presence of rock phosphate than in its absence. Additions of phosphorus and manure were shown to increase sulfur oxidation.

Shedd (47) made a comparative study of sulfur oxidation in the soil and in sand. That each soil has a definite sulfur oxidizing power has been demonstrated by Brown and Kellogg (11).

Halverson and Bollen (19) found that the application of sulfur tended to increase the sulfur oxidizing efficiency of the soil. These authors further showed that there was a correlation between the sulfur oxidizing power and the sulfate content of the soil.

Pfeiffer and Blanck (41) found that the increase in the sulfate content of the soil was proportional to the amount of sulfur added.

Ames and Richmond (2), Brown and Johnson (10), and Brioux and Guerbet (12) reported that calcium carbonate stimulated sulfur oxidation in the soil.

Neller (39) and Haynes (20) showed that sulfur was oxidized more rapidly under alkaline conditions and that a high concentration of salts did not prevent sulfur oxidation. This is in accord with the results obtained by Kelley and Thomas (28) and Samuels (46).

#### SULFUR OXIDATION IN RELATION TO BACTERIAL ACTIVITY

Brown and Kellogg (11), who first made a thorough study of sulfonation in soils, showed that the sulfur oxidation in soil was primarily brought about by bacterial action.

Joffe (25) stated that the oxidation of sulfur in the soil, while brought about chiefly by the activities of microorganisms, may be the result, in part, of chemical reactions. This has been pointed out by Kappen and Quensell (27). However, Boullanger (4) and Demolon (13, 14) emphasized the significance of the biological factor. They have shown that in sterilized soils the sulfur applied was not converted into sulfate as efficiently as in soils not sterilized.

Lipman (29) and his associates reported that elemental sulfur is oxidized by certain bacteria. Several types of sulfur oxidizing bacteria have been isolated and studied and the sulfur oxidizing process has been shown to occur in soils.

Shedd (48) found that sulfur oxidation did not proceed as rapidly in soil not inoculated as when the soil was inoculated with sulfur oxidizing organisms.

Gubin (18) suggested that different groups of sulfur organisms act on different sulfids. Among the iron sulfids, marcasite and ferrous sulfids are oxidized more rapidly than pyrite.

Several investigators have found, also, that the process of sulfur oxidation has some relation to the nitrifying bacteria and to the nitrogen cycle in the soil.

Boullanger and Dugardin (5) attributed the favorable action of sulfur on the soil to the stimulus produced on bacteria which were known to decompose nitrogenous matter with the production of ammonia.

Ames and Richmond (2) and Fife (15) stated that small amounts of elemental sulfur increased ammonification, but the process of nitrification was retarded.

Brown (6) found that sulfur oxidation affected the activities of the nitrifying organisms. Calcium carbonate when added to the soil frequently stimulated sulfur oxidation, and it also prevented the injurious effects of sulfur oxidation upon the nitrifying bacteria.

Pitz (42) stated that sulfur when applied to a silt loam soil increased the acidity of the soil and this tended to decrease the number of bacteria after a certain period. He further reported an increase in ammonification which was accompanied by a parallel decrease in nitrate formation.

Brioux and Guerbet (12) studied the transformation of sulfur in the soil, and the effect of carbohydrates, peptone and other nitrogenous material on the process of sulfur oxidation. They found that carbohydrates had a retarding effect, while peptone and certain other nitrogenous substances accelerated the process, so that in 30 days 82 per cent of the sulfur added was found to have been oxidized to sulfate.

Lipman and Joffe (30) and Brown (6) have shown that dextrose prevented the rapid oxidation of sulfur. The elimination of dextrose from the medium led to a more rapid oxidation.

#### SULFUR OXIDATION IN RELATION TO SOIL ACIDITY

The results of investigations have shown that the development of a strong acidity is caused by the continued application of sulfur, especially where large amounts are applied to soils (41, 42, 44).

Hibbard (21) and Lipman, McLean and Lint (33) claimed that the increase in the acidity of the soil was caused by the oxidation of the sulfur and phosphorus in the soil.

Tottingham and Hart (52) reported a gradual increase in the acidity in a garden soil composted with sulfur and rock phosphate. The acidity of the soil was found to be correlated with the amount of sulfur added. Similar results have been secured by Rudolfs (45) and Simon and Schollenberger (49).

Lipman, McLean and Lint (33), Neller (40) and Joffe and McLean (26) showed that applications of 200 or 500 pounds of sulfur did not materially change the hydrogen ion concentration through the season. Higher applications caused a decided increase in hydrogen ion concentration after the fourth to eighth weeks.

Gardner, Noll and Baker (16) stated that failures of crops at the Pennsylvania Agricultural Experiment Station Farm were mainly brought about by the strong acidity which developed as a result of the continued application of ammonium sulfate.

Adams (1) applied sulfur to Miami silt loam soil at the rate of 1,500, 2,000 and 3,000 pounds per acre and incubated the composts for a period of 30 days. The hydrogen ion concentration was found to have increased from 4.75 to 3.62 and 3.16, respectively.

Reimer and Tartar (43) believe that sulfur should not be applied to soils deficient in lime as it causes acidity to develop.

## I. EXPERIMENTAL

### STUDIES ON PLOT SOILS

This work was carried out on a series of plots under the five-year rotation of corn, oats, clover, wheat and alfalfa on the Agronomy Farm of the Iowa Agricultural Experiment Station. The soil is a typical Wisconsin drift and the plots include soils of the Carrington series, Carrington loam and Carrington fine sandy loam.

The treatments of the plots are as follows:

Plot No.	Treatment
912	Check
913	Manure
914	Manure + lime
915	Manure + lime + rock phosphate
916	Manure + lime + superphosphate
917	Check
918	Crop residue
919	Crop residue + lime
920	Crop residue + lime + rock phosphate
921	Crop residue + lime + superphosphate
922	Check

### *Methods of Treatments*

The various fertilizing materials are applied in amounts which are ordinarily used in practice. Manure is added at the rate of 10 tons per acre. Lime is applied at the rate of three and one-half tons per acre. The application is made in the spring preceding seeding of the clover crop. Rock phosphate is applied at the rate of 2,000 pounds per acre. The application is generally made in the fall and the phosphate is plowed under. The superphosphate is applied at the rate of 200 pounds per acre annually. It is usually applied in the spring and disced in.

The first sampling was made on August 19, and samples were taken at irregular intervals for several months. The sampling dates are given in table 1.

### *Method of Sampling*

The soil samples were taken by removing at least two inches of the surface soil from an area approximately 20 inches square. The soil was thoroughly stirred to a depth of from four to seven inches and the samples taken. Twenty small samples were drawn in this manner from each of the plots and well mixed to form composite samples, which were then placed in mason jars.



The samples were immediately taken to the laboratory and tests were made of the numbers of molds and bacteria, the moisture content, the reaction, and the sulfur oxidizing power of the soil. The quinhydrone electrode method was employed in the determination of the soil reaction.

#### *Determination of the Number of Bacteria in the Soil*

The number of bacteria present in the soil was determined by plate method, using Brown's modified albumen agar, which has the following composition:

Dextrose .....	10.00 gms.
Di-potassium phosphate ( $K_2HPO_4$ ) .....	0.50 gm.
Magnesium sulfate ( $MgSO_4 \cdot 7H_2O$ ) .....	0.20 gm.
Ferrie sulfate $Fe_2(SO_4)_3$ .....	Trace
Egg albumen .....	0.25 gm.
Agar .....	15.00 gms.
Distilled water .....	1000.00 cc.

The reaction was adjusted to pH 7.0.

The usual dilution method was employed, using physiological salt solution for the dilutions. Fifty grams of soil were shaken with 500 cc. of salt solution for five minutes, the dilutions made and the plates prepared. The plates were incubated at room temperature for one week, after which time the colonies were counted. The numbers of colonies secured on five plates were averaged.

#### *Determination of the Number of Molds in the Soil*

Waksman's synthetic acid agar was used in the determination of the number of molds. The medium was as follows:

Glucose .....	10.00 gms.
Peptone .....	5.00 gms.
$KH_2PO_4$ .....	1.00 gm.
$MgSO_4 \cdot 7H_2O$ .....	0.50 gm.
Agar .....	25.00 gms.
Distilled water .....	1000.00 cc.

The reaction was adjusted to a pH of 4.0 with normal sulfuric acid. The incubation period was three to four days.

#### *Results of the Determinations of the Numbers of Bacteria*

The results obtained in the bacterial counts on each of the 11 soils at the various samplings are shown in table 1.

It will be noted that the fluctuation in bacterial numbers in each of the soils was considerable. This was especially true with the manured soils, in which the rise and fall in the numbers were more evident than with the soils in the crop residue plots. The check plots, however, showed a more or less regular curve for the rise and fall in the numbers of bacteria.

TABLE 1. *Numbers of bacteria per gram of air-dry soil*

Plot	Dates of sampling								
	Aug. 19	Sept. 8	Sept. 22	Oct. 5	Oct. 20	Nov. 28	Dec. 23	Jan. 7	Average
912	2,285,000	2,830,000	3,170,000	2,985,000	1,780,000	2,275,000	3,180,000	3,375,000	2,735,000
913	4,730,000	6,410,000	3,405,000	5,270,000	2,290,000	3,290,000	2,760,000	5,900,000	4,248,000
914	2,610,000	3,629,000	2,825,000	2,935,000	1,305,000	4,070,000	3,070,000	6,080,000	3,315,000
915	4,930,000	6,530,000	2,955,000	4,560,000	1,760,000	3,720,000	3,590,000	6,890,000	4,378,400
916	6,420,000	6,640,000	4,670,000	5,960,000	2,875,000	4,140,000	3,790,000	6,750,000	5,155,600
917	3,375,000	3,890,000	2,730,000	3,410,000	1,572,000	1,885,000	3,540,000	3,730,000	3,016,000
918	4,240,000	4,760,000	2,585,000	3,750,000	1,910,000	2,680,000	3,086,000	4,760,000	3,471,400
919	3,495,000	4,340,000	4,356,000	3,710,000	1,669,000	3,330,000	3,979,000	4,050,000	3,616,200
920	4,140,000	5,060,000	3,856,000	3,840,000	2,089,000	2,890,000	3,770,000	4,330,000	3,748,000
921	4,460,000	5,450,000	4,160,000	5,390,000	1,632,000	3,025,000	3,299,000	4,680,000	4,012,000
922	2,875,000	3,080,000	3,445,000	3,980,000	1,650,000	2,275,000	3,525,000	4,520,000	3,168,750
Av.	3,960,000	4,774,460	3,468,820	4,162,730	1,860,190	3,050,990	3,326,270	5,005,910	

Considering the averages of the bacterial counts in all the soils at each sampling, it may be of interest to note that the numbers varied considerably. For instance, on August 19, when the first sampling was made, there were 3,960,000 bacteria per gram of soil. This was followed by an increase on September 8. At the third sampling there was a decrease, followed by an increase at the fourth sampling. At the fifth sampling, which was made on October 20, the bacterial numbers had dropped considerably. After that time the number gradually increased, and on January 7, when the last sampling was made, there were 5,005,910 bacteria per gram of soil. This suggests that seasonal condition and temperature affect to a marked extent the number of bacteria in the soil.

The data also indicate that the bacterial numbers present in the soil varied with the soil treatments. Comparing the bacterial counts in the soils in the manured plots, it will be noted that the lime, manure and superphosphate treated plot (916) showed the largest number of bacteria. Plot 915, which received applications of manure, lime and rock phosphate, was second. The soil in plot 913, which was treated with manure alone, showed a greater number than the soil in plot 914, which received manure and lime. In this case, it would appear that the addition of lime to the soil in plot 914 did not favorably influence the bacterial development.

It was found that the three check plots, 912, 917 and 922, showed lower numbers than any of the treated plots. The soils from plot 916, to which manure, lime and superphosphate were applied, contained a greater number of bacteria than that from plot 921, which was treated with crop residues, lime and superphosphate. Likewise, the soil in plot 915, which was treated with crop residues, lime and rock phosphate, contained greater bacterial numbers than the soil in plot 920, which received an application of crop residues, lime and rock phosphate.

The soil from plot 914, treated with manure and lime, showed a smaller number of bacteria than that from plot 919, which was treated with crop residues and lime. The difference, however, was not very significant. The soil in plot 913, with a treatment of manure alone, was found to contain more bacteria than the soil in plot 918, which received an application of crop residues alone.

When the soils from the manure and the crop residue plots are compared it is noted that the former showed a greater number of bacteria than the latter. It appears, therefore, that the manure had a greater effect on bacterial numbers than the crop residues exerted. This finding is in accord with the results secured by Brown (7) and Greaves and Carter (17).

### *Determination of the Numbers of Molds*

The results of the determination of the numbers of molds are presented in table 2. From these data it appears that in most cases the numbers fluctuated without much regard to seasonal conditions. When the mold counts of each of the 11 soils at the conclusion of the tests are compared it may be noted that the soil treatments did not seem to have a direct influence on the rise and fall of the numbers.

Comparing the mold content with the bacterial counts obtained on each of the soils, there seems to be some relationship between the development of these two groups of organisms. It appears that there was a rather defi-

TABLE 2. *Number of molds per gram of air-dry soil*

Plot No.	Dates of sampling								
	Aug. 19	Sept. 8	Sept. 22	Oct. 5	Oct. 20	Nov. 28	Dec. 23	Jan. 7	Average
912	315,000	271,900	240,100	210,000	189,000	227,500	273,000	349,000	259,500
913	499,000	376,000	361,900	285,900	335,000	306,100	253,000	385,000	350,273
914	369,900	275,000	239,000	228,200	206,500	302,500	239,000	374,500	279,325
915	350,000	331,500	426,000	344,500	384,800	431,000	247,000	371,900	380,837
916	375,000	303,000	412,000	472,000	380,000	379,000	263,500	270,000	356,850
917	348,500	279,000	395,500	286,000	281,200	341,000	274,200	410,000	326,925
918	512,000	343,100	303,000	341,000	314,500	289,000	240,000	405,000	343,450
919	470,000	303,500	290,100	292,000	289,000	264,000	261,000	345,000	313,075
920	326,600	348,500	330,000	214,500	307,900	266,000	251,800	351,000	299,537
921	435,000	379,000	348,500	284,000	315,000	267,000	261,500	316,000	350,750
922	276,900	285,800	255,500	398,000	263,900	238,500	238,500	309,000	283,262
Av.	388,900	363,290	325,600	314,200	296,980	303,055	254,770	373,310	



nite ratio between the numbers of molds and bacteria. For instance, the increase in the number of bacteria in each of the manured soils was accompanied by an increase in the mold content. In the crop residue treated soils, however, it is noted that as the numbers of bacteria increased, the molds decreased. In the manured soils there was greater development of both groups of organisms than in the crop residue treated soils.

### *Moisture Determinations*

Table 3 shows the results obtained from the determinations of the moisture content of the soils of the various plots. The data indicate that there were no great differences in the amount of moisture in the manured and the crop residue treated soils. The manured soils showed a somewhat lower moisture content and this difference was probably a result of the fact that these soils are lighter in texture than those in the crop residue plots. The addition of manure would be expected to increase the water-holding capacity of the soils, but did not bring about sufficient influence to offset the effect of the difference in texture.

According to these results the moisture content of the soil influenced the bacterial development to a large extent. The numbers of bacteria decreased with decreases in the moisture content of the soils in the various plots. The increases in the numbers of bacteria were also in most cases accompanied by an increase in moisture content. The number of molds, on the other hand, fluctuated without regard to the moisture in the soil. In fact, a gradual decrease in the mold counts occurred from the first to the fourth samplings, while there was an increase in the moisture content.

TABLE 3. *Moisture content of the soils*  
(Percentage)

Plot No.	Dates of sampling								Average
	Aug. 19	Sept. 8	Sept. 22	Oct. 5	Oct. 20	Nov. 28	Dec. 23	Jan. 7	
912	8.70	8.70	9.80	11.10	11.10	13.65	13.65	16.30	11.64
913	7.54	11.10	6.40	9.80	11.10	17.65	14.90	20.50	12.38
914	8.70	12.80	8.70	12.70	10.70	16.30	15.65	19.65	13.15
915	9.80	11.10	9.80	11.10	9.80	16.30	12.40	20.50	12.60
916	11.10	8.70	8.70	12.40	11.10	14.90	14.90	20.50	12.80
917	12.40	11.10	13.70	9.80	12.40	17.65	14.90	20.50	14.06
918	8.70	11.10	12.40	13.65	12.40	16.30	13.65	19.00	13.40
919	9.80	12.40	12.40	12.40	11.10	14.90	13.65	19.00	13.21
920	8.70	12.40	13.70	13.65	9.80	16.30	14.98	17.65	13.40
921	8.70	11.10	12.40	12.40	8.70	16.30	13.65	17.65	12.61
922	7.40	9.80	11.10	13.65	9.80	13.65	13.65	19.00	12.38
Av.	9.22	10.94	10.80	12.06	10.72	15.81	14.18	19.11	

### *Hydrogen Ion Concentration Determinations*

The results of the hydrogen ion determinations as measured by the

quinhydrone electrode method are given in table 4. The data show that there was no significant variation in the reaction of the soils in each of the 11 plots.

Comparing the average results on the different dates of samplings, it is noted that the seasonal effects were not marked. All the manured soils, plots 913, 914, 915 and 916, showed an average pH of 6.09, as compared with 6.01 for the crop residue treated soils, plots 918, 919, 920 and 921, for the entire sampling period. It would appear, therefore, that the reaction of these soils was not definitely correlated with the development of bacteria and molds in the soils.

### *Sulfur Oxidation Experiments*

The method used was as follows:

Six one-hundred-gram portions of each of the soils under investigation were placed in tumblers. Two tumblers were used as checks. To each of two tumblers, one gram of elemental sulfur was added. In two tumblers the soils were treated with four grams of sodium thiosulfate. The tumblers were covered with tin covers and incubated at room temperature for six weeks at a moisture content of 50 per cent saturation.

TABLE 4. *Hydrogen ion concentration of the soils—(pH)*

Plot	Dates of sampling								
No.	Aug.19	Sept. 8	Sept. 22	Oct. 5	Oct. 20	Nov. 28	Dec. 23	Jan. 7	Av.
912	6.20	5.90	6.00	5.90	6.00	5.90	5.90	5.80	5.95
913	6.00	6.20	6.00	6.00	6.00	6.20	6.10	6.00	6.06
914	6.00	6.20	6.00	6.00	6.10	6.00	6.00	5.90	6.03
915	5.90	6.20	5.90	6.10	6.20	6.10	6.10	6.00	6.06
916	6.20	6.40	6.20	6.20	6.00	6.20	6.30	6.10	6.20
917	6.00	6.20	6.20	6.00	6.00	6.20	6.20	6.00	6.10
918	6.30	6.00	6.00	6.00	5.90	6.00	6.00	5.80	6.00
919	6.20	6.20	6.20	5.90	5.90	6.00	6.00	5.80	6.03
920	6.00	5.90	6.20	6.80	6.10	5.90	5.80	6.00	5.96
921	6.30	6.00	6.00	6.00	6.00	6.00	5.90	6.00	6.03
922	6.30	6.00	5.90	6.00	6.00	5.90	6.00	5.90	6.00
Av.	6.13	6.11	6.06	5.91	6.02	6.04	6.03	5.57	

The amount of sulfates was then determined by the use of a sulfur photometer.

### *Results of the Experiments on Sulfur Oxidation*

The average results presented in table 5 are expressed in milligrams of sulfur as sulfate per 100 grams of air-dry soil. The results in column A were secured with the elemental sulfur treatment and column B with the sodium thiosulfate application.

A study of the figures shows that all the soils in the variously treated plots differed in their sulfur oxidizing efficiency. A greater sulfur oxidizing power was observed with the use of sodium thiosulfate than with the ele-

mental sulfur. In the later discussion of the results, unless otherwise stated, only the figures given in column A are considered.

Comparing the amounts of sulfates which were produced in each of the 11 soils at the end of the sampling period, it is evident that the soil treatments exerted an influence on the sulfur oxidizing power of the soil. Considering only the manured plots, it will be noted that the soil from the manure, lime and superphosphate treated plot (916) was capable of producing the greatest amount of sulfate. Plot 915, treated with manure, lime and rock phosphate, showed a greater sulfur oxidizing power than the soil from the manure and lime treated plot (914). With the manure application

TABLE 5. *Sulfur oxidation experiments*

Plot No.	Milligrams of sulfur as sulfate per 100 grams of soil from various plots**									
	Aug. 19		Sept. 8		Sept. 22		Oct. 5		Oct. 20	
	A*	B*	A	B	A	B	A	B	A	B
912	42.00	62.25	62.13	94.88	108.13	115.50	109.00	144.38	109.25	126.00
913	79.88	100.30	95.50	115.88	92.50	99.63	103.69	116.88	109.75	115.00
914	71.50	100.02	74.50	105.25	87.13	94.38	93.13	108.13	106.00	143.00
915	76.75	116.00	83.63	120.88	89.88	104.63	106.63	115.00	108.50	119.25
916	89.88	124.38	84.88	92.50	108.13	118.13	102.50	112.50	105.75	126.25
917	100.25	111.88	95.63	124.38	103.63	113.13	104.25	116.25	116.25	118.75
918	100.88	151.00	98.13	110.00	104.25	115.88	109.75	116.75	115.00	119.50
919	101.38	126.25	95.75	130.00	113.75	119.38	114.00	118.50	118.75	124.24
920	115.88	124.38	115.88	128.13	109.38	114.13	115.00	119.00	119.25	132.18
921	130.00	137.13	121.88	148.75	103.50	109.38	119.50	123.00	126.00	140.25
922	102.50	121.00	110.00	130.00	104.25	145.63	124.50	133.80	124.75	133.50
Av.	91.90	115.85	94.36	118.24	103.14	113.61	111.04	120.38	126.59	137.27

Plot No.	Nov. 28		Dec. 23		Jan. 7		Average	
	A	B	A	B	A	B	A	B
912	130.50	155.00	143.13	167.38	169.13	196.25	109.16	132.72
913	137.75	138.75	150.63	187.25	173.38	226.63	117.88	137.54
914	146.75	155.13	182.75	201.38	201.13	209.00	120.36	139.54
915	159.88	178.00	191.38	204.88	185.13	191.75	125.22	143.80
916	161.50	168.50	189.00	207.75	188.13	209.63	128.72	144.95
917	137.63	154.88	184.25	203.13	196.88	228.38	129.85	146.35
918	138.00	160.63	184.25	218.75	201.75	225.50	131.50	152.13
919	156.13	177.38	196.25	226.88	183.25	227.25	134.90	156.24
920	154.25	169.75	201.75	233.75	210.12	236.88	142.69	157.27
921	132.00	157.38	207.38	216.90	208.88	225.38	143.61	157.26
922	131.50	142.00	206.88	220.88	214.38	227.25	137.84	156.75
Av.	144.17	159.76	185.24	208.06	193.83	217.53		

\*A—Elemental sulfur treatment.

B—Sodium thiosulfate treatment.

\*\*Results given are the average of duplicates.

alone, plot 913, the sulfur oxidizing power was the lowest. This relation between the soil treatments and the sulfur oxidizing power of the soils in the manured plots was found to correspond with that found for the soils from the crop residue treated plots.

TABLE 6. *Average results of moisture, reaction, numbers of microorganisms and sulfur oxidation*

Plot No.	Percentage H <sub>2</sub> O	pH	Numbers of microorganisms		Mgm. sulfur as sulfates*	
			Bacteria	Molds	A	B
912	11.64	5.95	2,735,000	259,500	109.16	132.72
913	12.38	6.06	4,248,000	350,237	117.88	137.54
914	13.15	6.03	3,315,500	279,325	120.36	139.54
915	12.60	6.06	4,378,400	380,837	125.22	143.80
916	12.80	6.20	5,155,600	356,850	128.17	144.95
917	14.06	6.10	3,016,500	326,925	129.85	146.35
918	13.40	6.00	3,471,400	343,450	131.50	152.13
919	13.21	6.03	3,616,200	313,075	134.90	156.24
920	13.40	5.96	3,748,000	299,537	142.69	157.27
921	12.61	6.03	4,012,000	350,750	143.61	157.26
922	12.38	6.00	3,168,750	283,262	137.84	156.75

\*A, 1 gm. of elemental sulfur used in the treatment.

B, 4 gms. of sodium thiosulfate used in the treatment.

#### *Average Results of Studies on Plot Soils*

The average results of all the tests on these soils are shown in table 6 and the relations of the numbers of bacteria and molds to sulfur oxidation are shown in figure 1. From these data it appears that there was a correlation between the sulfur oxidizing power of the soil and bacterial numbers present. In all the soils in each of the two groups of plots, with the exception of that in plot 914, there was a definite relation between bacterial numbers and the sulfate production.

Comparing the two groups of plots, it will be noted that in general the crop residue treated soils produced more sulfate than the manured soils regardless of the larger bacterial numbers in the latter. The relative increases in the amounts of sulfates produced in the soil from the crop residue plots did not correspond with an increase in the numbers of bacteria. This being the case, it would seem that the textural difference in the soils may possibly have played an important part in stimulating the sulfur oxidizing action of the organisms in the soil.

Considering the relation of mold content to sulfate production in the soil, it is apparent that the numbers of molds did not run parallel with the amount of sulfates produced. In fact, the number of molds in the soil fluctuated with little regard to sulfate production.

The results secured from the manured soils showed when any relation between the amount of sulfate produced and the number of molds was indicated, the relative number of bacteria in proportion to the number of molds was increased. In the crop residue treated soils, however, this relationship did not appear.



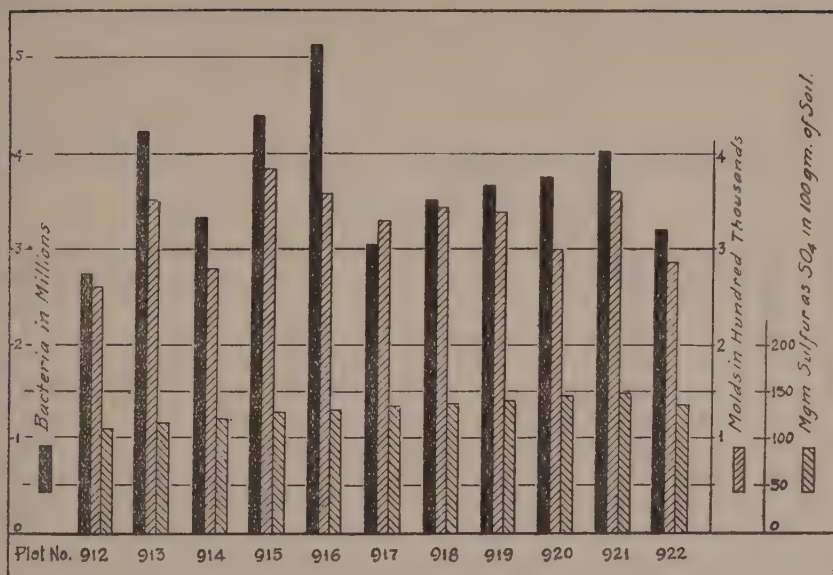


Fig. 1. Average numbers of bacteria and molds and mgm. sulfur as sulfates

The largest numbers of bacteria were found in the soil from plot 916. The largest numbers of molds appeared in the soil from plot 915, but the greatest sulfur oxidizing power was shown in the soil from plot 921. The check soil (912) was the lowest in numbers of organisms and in sulfur oxidizing power.

## II. LABORATORY STUDIES ON SULFUR OXIDATION

### A. *The Influence of Calcium Carbonate and Dextrose on Sulfur Oxidation*

This experiment was planned to determine the effect of calcium carbonate and dextrose on the sulfur oxidizing power of the soil.

The soil used in this experiment was secured from the manure, lime and superphosphate treated plot (916). The soil was air-dried, sieved and treated with the materials as indicated in table 7. At the end of the incubation period, the amount of sulfur as sulfate was determined, using the photometric method. The results, calculated in milligrams of sulfur as sulfate in 100 grams of air-dry soil, are given in the table.

### *Results*

From the average figures of the duplicate treatments shown in table 7, it may be noted that there were significant variations in the amount of sulfate produced.

The tests using sodium thiosulfate showed that when dextrose was applied 115.25 mgm. of sulfur as sulfate were produced, while the soil without

TABLE 7. *The effect of 0.3 gram of calcium carbonate on the oxidation of sodium thiosulfate and sulfur in soil treated with one per cent of dextrose*

Lab. No.	Treatment				Mgm. sulfur as sulfate in 100 grams of air-dry soil	
	Na <sub>2</sub> S <sub>2</sub> O <sub>5</sub>	Sulfur	Dextrose	CaCO <sub>3</sub>		
1	Check	-----	-----	-----	Trace	Av.
2	"	-----	-----	-----	"	Trace
3	4 gms.	-----	-----	-----	172.30	
4	"	-----	-----	-----	174.10	173.20
5	"	-----	1 gm.	-----	114.62	
6	"	-----	"	-----	115.87	115.25
7	"	-----	"	0.3 gm.	210.82	
8	"	-----	"	"	203.56	207.19
9	-----	1 gm.	-----	-----	62.35	
10	-----	"	-----	-----	59.33	60.34
11	-----	"	1 gm.	-----	103.25	
12	-----	"	"	-----	108.25	105.75
13	-----	"	"	0.3 gm.	161.38	
14	-----	"	"	"	166.38	163.88

the dextrose produced 173.20 mgm. The dextrose, therefore, gave a decrease of 57.95 mgm. There is certainly a definite retarding influence of dextrose on the oxidation of sulfur in the soil when tested with sodium thiosulfate. It may be noted that this observation is in accord with the conclusions of Brown (6) and Lipman and Joffe (30). These authors claim that dextrose prevents the rapid oxidation of sulfur in the soil.

The soil which was treated with dextrose and calcium carbonate showed a production of 207.19 mgm. of sulfate or an increase of 91.94 mgm. over the 115.25 mgm. produced in the soil treated with dextrose alone. The difference in the amount of sulfates produced with these two different treatments is certainly due to the influence of the addition of calcium carbonate on the sulfur oxidizing power of the soil, or to its effect in overcoming the injurious influence of the dextrose.

Lime, according to the findings of Brown and Johnson (10) and Neller (39) increased the sulfur oxidizing power of the soil.

The results secured with the elemental sulfur were parallel with those obtained with sodium thiosulfate application, but the figures were smaller.

#### *B. Influence of Sulfur and Lime Applications on the Nitrifying Power of the Soil*

In the review of the literature it was noted that sulfur additions had been observed to cause a retarding influence on the nitrifying efficiency of the soil. This experiment was carried out in order to determine the effects, if any, of various additions of sulfur on the nitrifying power of the soil. It was also considered desirable, if it is true that sulfur applications exert a toxic effect on the nitrifying power of the soil, to determine whether or not the addition of lime would be of value in preventing the toxic action of sulfur.

TABLE 8. *The effect of different amounts of sulfur with and without lime on the nitrifying power of the soil*

Lab. No.	30 mgm. N (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Treatment		Mgm. nitrogen as nitrate in 10 gms. of air-dry soil	
		Sulfur gram	Calcium carbonate		
1	Check	-----	-----	6.88	Av.
2	"	-----	-----	6.66	6.77
3	30 mgm.N	-----	-----	16.00	
4	"	-----	-----	15.40	15.70
5	"	0.025	-----	10.50	
6	"	"	-----	10.80	10.65
7	"	0.050	-----	9.75	
8	"	"	-----	9.52	9.64
9	"	0.100	-----	9.09	
10	"	"	-----	9.52	9.31
11	"	0.500	-----	8.69	
12	"	"	-----	8.12	8.41
13	"	1.000	-----	7.84	
14	"	"	-----	7.91	7.38
15	"	2.000	-----	6.66	
		"	-----	6.89	6.78
17	"	0.025	210 mgm.	18.18	
18	"	"	"	17.65	17.92
19	"	0.050	"	12.50	
20	"	"	"	12.10	12.30
21	"	0.100	"	11.42	
22	"	"	"	11.77	11.60
23	"	0.500	"	9.09	
24	"	"	"	8.90	9.00
25	"	1.000	"	8.00	
26	"	"	"	8.23	8.12
27	"	2.000	"	7.69	
28	"	"	"	7.25	7.47

Using soil from plot 918 (crop residue plot) cultures were prepared in the same way as in the sulfur oxidation experiment. The treatments are shown in table 8. After the incubation period, which lasted for four weeks, the nitrogen as nitrate was determined colorimetrically by the use of the phenoldisulfonic acid method. The results, which are expressed in milligrams of nitrogen as nitrate in 100 grams of air-dry soil, are given in table 8.

### Results

Considering the averages of the results of the duplicate determinations on all the samples, as shown in the table, it will be noted that the sulfur applications tended to cause a depressing effect on the nitrifying power of the soil.

It may also be observed that larger amounts of sulfur brought about greater decrease in nitrate production. Increasing the amount of the sulfur applications, however, did not seem to bring about a proportional decrease in the amount of nitrates produced.

The addition of sulfur to the soil, as pointed out by Martin (37), Neller (40) and Reimer and Tartar (43), leads to an increase in soil acidity caused by the oxidation of the sulfur to sulfuric acid. Lipman, McLean and Lint (33) also showed that the larger applications of sulfur caused a decided increase in the hydrogen ion concentration of the soil. The oxidation of elemental sulfur in the soil, as shown by Ames and Richmond (2) and Brown (6), depressed the activities of nitrifying bacteria.

If the suggestions of these authors are accepted, it would seem, therefore, that the decrease in the amount of nitrate produced, which indicates a retarded nitrifying power of the soil, may be attributed to the depressing effect of the acidity produced from the oxidation of the sulfur on the activities of the nitrifying organisms.

The sulfur-lime treated soils were found to be generally higher in their nitrifying power, as evidenced by the greater amount of nitrate produced, than those in which sulfur was applied alone. It may be noted, however, that when the sulfur applications were increased, the amount of calcium carbonate added had very little effect upon the rate of nitrate production in the soil. The increase in the amount of nitrate produced was undoubtedly a result of the stimulating influence of calcium carbonate upon the activities of nitrifying organisms in the soil, or to a removal of injurious acidity. Brown (6) showed a definite influence of lime in reducing the injurious effects of additions of sulfur on the nitrifying bacteria.

### *C. The Effect of the Various Amounts of Sulfur on the Nitrifying and Sulfur Oxidizing Powers of the Soil*

In the previous experiment it was indicated that the oxidation of sulfur in the soil may limit the nitrifying process because of an increase in soil acidity. The following test was planned to determine the relative influence of the application of various amounts of elemental sulfur, with and without the addition of lime, upon the nitrification and sulfur oxidation in the soil.

In this study soil taken from plot 921 that has been treated with crop residues, lime and superphosphate was used. The cultures were prepared in the usual way and the various treatments were made. The cultures were each provided with 30 milligrams of nitrogen as ammonium phosphate and were incubated at room temperature for a period of five weeks at a moisture content of 50 per cent saturation.

After incubation, the hydrogen ion concentration of each of the soils was determined. The photometric method was employed for the sulfate determination and the phenoldisulfonic acid method was used in the determination of nitrates. The results are given in table 9.

### *Results*

From the figures presented in table 9 it will be noted that increasing the sulfur applications brought about corresponding increases in the sulfate production and in the hydrogen ion concentration, which were not proportional to the amounts of sulfur applied.



TABLE 9. The effect of the various amounts of sulfur with and without lime on the nitrifying and sulfur oxidizing powers of the soil

Lab. No.	Treatment		Hydrogen ion * Concentrations	Increase in av. of pH values	Mgm. sulfur as sulfate in 100 grams soil		Aver. age sulfur oxidized	Mgm. nitrogen as nitrate in 100 grams soil		Aver. age nitrogen oxidized	
	(NH <sub>4</sub> ) <sub>2</sub> PO <sub>4</sub> 30 mgm.	Elemental sulfur			CaCO <sub>3</sub> 210 mgm.	Av.		Petg.	Av.		Petg.
1	Check	.....	6.10	.....	Trace	.....	.....	6.34	.....	.....	
2	"	.....	6.06	.....	"	.....	.....	6.42	.....	.....	
3	(NH <sub>4</sub> ) <sub>2</sub> PO <sub>4</sub>	.....	6.18	.....	"	.....	.....	18.18	.....	.....	
4	"	.....	6.00	.....	"	.....	.....	19.60	.....	.....	
5	"	0.025 gm.	5.20	.....	11.25	.....	.....	17.65	.....	.....	
6	"	"	5.25	14.29	12.25	47.00	47.00	15.40	.....	.....	
7	"	0.050 gm.	4.88	.....	12.74	.....	.....	12.50	.....	13.20	
8	"	"	4.93	19.38	12.74	.....	.....	.....	.....	.....	
9	"	0.100 gm.	4.38	.....	19.20	.....	.....	10.50	.....	10.00	
10	"	"	4.76	24.96	19.25	19.23	19.23	9.75	10.13	8.10	
11	"	0.500 gm.	3.08	.....	95.75	.....	.....	8.33	.....	.....	
12	"	"	3.28	48.12	95.00	19.06	19.06	8.33	8.33	6.66	
13	"	1.000 gm.	3.32	.....	98.75	.....	.....	7.69	.....	.....	
14	"	"	3.12	47.13	98.75	98.75	9.88	7.84	7.77	6.62	
15	"	2.000 gm.	3.18	.....	120.63	.....	.....	7.14	.....	.....	
16	"	"	3.17	47.78	123.75	.....	6.11	7.06	7.10	5.68	
17	"	0.025 gm.	6.36	.....	17.35	.....	.....	20.00	.....	.....	
18	"	"	6.33	—43	17.50	17.43	67.70	18.18	19.09	15.27	
19	"	0.050 gm.	6.23	.....	19.00	.....	.....	16.60	.....	.....	
20	"	"	6.23	—23	19.25	19.13	36.25	16.00	16.30	13.04	
21	"	0.100 gm.	5.93	.....	.....	.....	.....	.....	.....	.....	
22	"	"	5.88	3.12	20.10	20.10	20.10	15.40	14.85	11.88	
23	"	0.500 gm.	5.85	.....	100.00	.....	.....	12.50	.....	.....	
24	"	"	5.85	3.94	98.25	99.13	19.82	11.75	12.13	9.70	
25	"	1.000 gm.	5.25	.....	136.00	.....	.....	9.75	.....	.....	
26	"	"	.....	13.79	134.35	135.18	13.52	9.09	9.42	7.53	
27	"	2.000 gm.	4.58	26.93	140.35	.....	6.58	8.33	8.32	6.65	
28	"	"	4.33	.....	123.00	131.68	.....	8.30	.....	.....	

\* Figures represent pH.

\*Figures represent pH.

The rate of sulfur oxidation in the soil varied with the sulfur applications. It was found that sulfur oxidation occurred most vigorously and efficiently when sulfur was added in the smallest amount; and where it was applied in the largest amount the relative amount of sulfur oxidized was the smallest (6.11 per cent). The results showed further that when 25 milligrams of elemental sulfur were used, 46 per cent was oxidized. The addition of 100 and 500 milligrams of sulfur to the soil brought about a much smaller percentage oxidized, but a similar amount for the two additions (19.23 and 19.06 per cent, respectively). A further increase in the sulfur application caused a marked decrease in the rate of oxidation. When one and two grams of sulfur were added, the relative amounts oxidized did not vary materially.

From the results it seems evident that while the soil showed a definite sulfur oxidizing efficiency, the sulfur oxidation seemed to reach a maximum with certain amounts of sulfur, and when this point was reached, a further addition depressed the sulfur oxidizing power of the soil.

While the process of nitrification in the soil occurred in spite of the increasing development of soil acidity brought about by the sulfur oxidation, it decreased gradually as sulfur oxidation and soil acidity increased. The most favorable condition for nitrification was found when sulfur was added in the smallest amount. There was a gradual decrease in the amount of nitrate produced as the amount of sulfur added was increased.

The addition of calcium carbonate not only stimulated the oxidation of sulfur in the soil, but also tended to reduce the depressing influence of the acidity on the nitrification process.

#### DISCUSSION AND SUMMARY

In this work studies have been made on sulfur oxidation, the numbers of bacteria and molds, the reaction and the moisture content in soil from plots variously treated.

The data in table 1 indicated that bacterial numbers varied with the soil treatments. The occurrence of bacteria in the soil was also influenced by seasonal conditions.

Comparing the two groups of plots, the manured and the crop residue plots, it appears that there was a greater number of bacteria in the manured soils than in the crop residue treated soils. The results seemed to indicate that the manure increased the bacterial numbers more than did the crop residue treatment.

A study of the data presented in table 2 shows that the soil treatments and the seasonal conditions did not seem to have a significant influence on the mold occurrence in the soil.

When a comparison is made of the mold and the bacterial counts secured from each of the manured soils, it is found that there was some relation between the development of these two groups of organisms.

The data given in tables 3 and 4 indicate that the moisture content of the soils exerted a greater influence on the bacteria than it did on the molds. The hydrogen ion concentration of the soils studied did not show a definite influence on either the molds or the bacteria.

The results of the sulfur oxidation studies show that the soils had a definite sulfur oxidizing efficiency.

Comparing the results on the manured and the crop residue treated soils, it may be noted that there was greater oxidation of sulfur in the soils treated with the crop residues than in those receiving manure, although the latter had greater numbers of bacteria. This difference has been attributed in part, at least, to the textural condition of the soils. It may be seen also that the numbers of molds did not run parallel with the amount of sulfates produced. Nevertheless, a greater sulfur oxidizing power of the soil was observed when the phosphate materials were added. In both groups of plots, it was found that the rock phosphate had somewhat less effect than the superphosphate; the difference, however, was not pronounced.

A definite correlation between the numbers of molds and bacteria and the sulfate production has also been shown. In the manured plots it was found that increases in the sulfate production were accompanied by increases in the numbers of bacteria. The numbers of molds did not increase with sulfate production, however. Dextrose was found to have a retarding influence on sulfur oxidation in the soil. Lime, when added to the soil, increased the sulfur oxidizing power and reduced the retardation brought about by the dextrose.

The nitrifying power of the soil was found to be appreciably depressed by the addition of sulfur. Greater decreases in the nitrate production occurred with the larger sulfur applications. The addition of sulfur increased the acidity of the soil and this retarded the nitrification process.

The rate of sulfur oxidation in the soil varied with the amounts of sulfur added. A marked decrease in the rate of sulfur oxidation was brought about by too large an addition of sulfur.

#### CONCLUSIONS

1. The soils studied showed a definite sulfur oxidizing efficiency which varied with the different soil treatments.
  - a. Manure treatments appeared to cause a slight increase in sulfur oxidation.
  - b. Lime applications apparently brought about a small increase in the sulfur oxidizing efficiency of the soil.
  - c. The phosphate treatments caused a still greater increase in the sulfur oxidizing power of the soil; superphosphate seemed to be slightly more effective than rock phosphate.
2. A correlation was found between the sulfur oxidizing power of the soils and the numbers of bacteria present.
3. The numbers of molds in the soils were not correlated with the sulfate production.
4. Dextrose, when added to the soils, retarded the process of sulfur oxidation.
5. The rate of sulfur oxidation in the soil was influenced by the various amounts of sulfur added in the tests.
6. Sulfur oxidation was most vigorous in the presence of the smallest sulfur applications.

7. The application of sulfur to the soil brought about an increased hydrogen ion concentration.
8. The addition of sulfur retarded the process of nitrification. The highest concentrations of sulfur used did not inhibit the process.
9. Lime not only increased the sulfur oxidizing power of the soil, but also tended to decrease the injurious effect of dextrose on sulfur oxidation.

## BIBLIOGRAPHY

1. ADAMS, H. R.  
1924. Some effects of sulfur on crops and soils. *Soil Sci.*, **18**:111-115.
2. AMES, J. W., AND T. E. RICHMOND  
1918. Sulfonation in relation to nitrogen transformation. *Soil Sci.*, **1**:311-321.
3. BEIJERINCK, M. W.  
1904. Ueber die Bakterien, welche sich im Dunkeln mit Kohlensäure als Kohlenstoffquelle ernähren können. *Centbl. Bakt. II.*, **11**:593-599.
4. BOULLANGER, E.  
1912. L'action du soufre en fleur sur la vegetation. *Acad. Sci. (Paris)*, **154**:369-370.
5. BOULLANGER, E., AND M. DUGARDIN  
1912. Mecanisme de l'action fertilisante du soufre. *Compt. Rend. Acad. Sci. (Paris)*, **155**:327-329.
6. BROWN, H. D.  
1923. Sulfonation in pure and mixed cultures with special reference to sulfate production, hydrogen-ion concentration and nitrification. *Jour. Amer. Soc. Agron.*, **15**:350-382.
7. BROWN, P. E.  
1913. Bacteriological studies on field soils: III. The influence of barnyard manure. *Iowa Agr. Exp. Sta. Res. Bull.* **13**.
8. BROWN, P. E., AND A. R. GWINN  
1917. Effect of sulfur and manure on availability of rock phosphate in soil. *Iowa Agr. Exp. Sta. Res. Bull.* **43**.
9. BROWN, P. E., AND W. V. HALVERSON  
1919. Effects of seasonal conditions and soil treatment on bacteria and molds in soils. *Iowa Agr. Exp. Sta. Res. Bull.* **56**.
10. BROWN, P. E., AND H. W. JOHNSON  
1916. Studies in sulfonation. *Soil Sci.*, **1**:339-362.
11. BROWN, P. E., AND E. H. KELOGG  
1914. Sulfonation in soils. *Iowa Agr. Exp. Sta. Res. Bull.* **18**.
12. BRIOUX, CH., AND M. GUERBET  
1913. Evolution du soufre dans le sol, etude sur son oxydation. *Comp. Rend. Acad. Sci. (Paris)*, **156**:1476-1479.
13. DEMOLON, A.  
1912. Sur l'action fertilisante du soufre. *Compt. Rend. Acad. Sci. (Paris)*, **154**:524-526.
14. DEMOLON, A.  
1914. Recherches sur l'action fertilisante du soufre. *Compt. Rend. Acad. Sci. (Paris)*, **156**:725-728.
15. FIFE, J. M.  
1926. The effect of sulfur on the micro-flora of the soil. *Soil Sci.*, **12**:245-252.



16. GARDNER, F. D., C. F. NOLL AND P. S. BAKER  
1917. Thirty-five years' results with fertilizers. Penn. Agr. Exp. Sta. Bull. 146.
17. GREAVES, J. E., AND E. J. CARTER  
1916. Influence of barnyard manure and water upon the bacterial activities of the soil. Jour. Agr. Res., 6:889-926.
18. GUBIN, B. M.  
1926. On the oxidation of sulfur and sulfides by soil bacteria. (Russian) Vestnik Bakt. Agron. Sta., 24:52-74. Original not examined. Abst. in Waksman, S. A., 1927. Principles of soil microbiology. Williams & Wilkins Co., Baltimore, Md.
19. HALVERSON, W. V., AND W. B. BOLLEN  
1923. Studies on sulfur oxidation in Oregon soils. Soil Sci., 16:479-490.
20. HAYNES, J. D.  
1928. The rate of availability of various forms of sulfur fertilizers. Soil Sci., 25:447-454.
21. HIBBARD, P. L.  
1919. Changes in composition of the soil and of the water soluble extract of the soil following addition of manure. Soil Sci., 7:259-272.
22. HIBBARD, P. L.  
1921. Sulfur for neutralizing alkali soil. Soil Sci., 11:385-387.
23. JACOBSEN, H. C.  
1912. Die Oxydation von elementaren Schwefel durch Bakterien. Folia Microb., 1:487-496.
24. JACOBSEN, H. C.  
1914. Die Oxydation von Schwefelwasserstoff durch Bakterien. Folia Microb., 3:155-162.
25. JOFFE, J. S.  
1922. Preliminary studies on the isolation of sulfur oxidizing bacteria from sulfur-floats-soil composts. Soil Sci., 13:161-172.
26. JOFFE, J. S., AND H. C. MCLEAN  
1922. A note on oxidation of sulfur in Oregon soils. Soil Sci., 14:217-221.
27. KAPPEN, H., AND E. QUENSELL  
1915. Ueber die Umwandlungen von Schwefel und Schwefelverbindungen im Ackerboden, ein Beitrag zur Kenntnis des Schwefelkreislaufes. Landw. Vers. Stat., 86:1-34.
28. KELLEY, W. P., AND E. E. THOMAS  
1923. The removal of sodium carbonate from soils. Calif. Agr. Exp. Sta. Tech. Paper 1.
29. LIPMAN, J. G.  
1923. Recent investigations on the oxidation of sulfur by microorganisms. Jour. Indus. Engin. Chem., 15:404.
30. LIPMAN, J. G., AND J. S. JOFFE  
1920. The influence of initial reaction on the oxidation of sulfur and the formation of available phosphates. Soil Sci., 10:327-332.
31. LIPMAN, J. G., AND H. C. MCLEAN  
1917. Vegetation experiments on the availability of treated phosphates. Soil Sci., 4:337-342.
32. LIPMAN, J. G., AND H. C. MCLEAN  
1918. Experiments with sulfur phosphate composts conducted under field conditions. Soil Sci., 5:243-250.

33. LIPMAN, J. G., H. C. McLEAN AND H. C. LINT  
1916. Sulfur oxidation in soils and its effects on the availability of mineral phosphates. *Soil Sci.*, 2:499-538.
34. LIPMAN, J. G., A. L. PRINCE AND A. W. BLAIR  
1920. The influence of varying amounts of sulfur in the soil on crop yields, hydrogen-ion concentration, lime requirements and nitrate formation. *Soil Sci.*, 12:197-207.
35. LIPMAN, J. G., S. A. WAKSMAN AND J. S. JOFFE  
1924. The oxidation of sulfur by soil microorganisms. *Soil Sci.*, 12:475-489.
36. MARES, M. H.  
1869. Des transformations que subit le soufre en poudre (fleur de soufre et soufre triture) quand il est repandu sur le sol. *Comp. Rend. Acad. Sci. (Paris)*, 69:974-997.
37. MARTIN, W. H.  
1920. The relation of sulfur to soil acidity and the control of potato scab. *Soil Sci.*, 9:393-408.
38. McLEAN, H. C.  
1918. The oxidation of sulfur by microorganisms in its relation to the availability of phosphates. *Soil Sci.*, 5:251-290.
39. NELLER, J. R.  
1920. The oxidizing power of soil from lime and unlimed plots and its relation to other factors. *Soil Sci.*, 10:29-39.
40. NELLER, J. R.  
1925. Sulfonation and its effects upon the oxidation of organic matter in eastern Washington soils. *Jour. Amer. Soc. Agron.*, 17:40-48.
41. PFEIFFER, TH., AND E. BLANCK  
1913. Beitrag zur Wirkung des Schwefels auf die Pflanzenproduction, sowie zur Anpassung der Ergebnisse von Feldversuchen an das Gaussche Fehlerwahrscheinlichkeitsgesetz. *Landw. Vers. Stat.*, 83:359-383.
42. PITZ, W.  
1916. Effect of elemental sulfur and of calcium sulfate on certain of the higher and lower plant life. *Jour. Agr. Res.*, 5:771-780.
43. REIMER, F. C., AND H. V. TARTAR  
1919. Sulfur as a fertilizer for alfalfa in southern Oregon. *Ore. Agr. Exp. Sta. Bull.* 163.
44. REYNOLDS, E. B., AND A. H. LEIDIGH  
1922. Sulfur as a fertilizer for cotton. *Soil Sci.*, 14:435-440.
45. RUDOLFS, W.  
1921. Influence of sulfur oxidation upon the growth of soybeans and its effect on bacterial flora of soil. *Soil Sci.*, 14:247-264.
46. SAMUELS, C. D.  
1927. The oxidation of sulfur in alkali soils and its effect on the replaceable bases. *Calif. Agr. Exp. Sta. Hilgardia* 3:No. 1.
47. SHEDD, O. M.  
1914. The relation of sulfur to soil fertility. *Ky. Agr. Exp. Sta. Bull.* 188.
48. SHEDD, O. M.  
1919. Effect of oxidation of sulfur in soils on the solubility of rock phosphate and on nitrification. *Jour. Agr. Res.*, 18:329-345.
49. SIMON, R. H., AND C. J. SCHOLLENBERGER  
1925. The rate of oxidation of different forms of elemental sulfur. *Soil Sci.*, 20:443-449.

50. STARKEY, R. L.  
1924. Concerning the physiology of *Thiobacillus thiooxidans*, an autotrophic bacterium oxidizing sulfur under acid conditions. Jour. Bact., 10:135-195.
51. STEPHENSON, R. E.  
1925. Relation of fineness of grinding to rate of sulfur oxidation in soils. Soil Sci., 21:489-494.
52. TOTTINGHAM, W. E., AND E. B. HART  
1920. Sulfur and sulfur composts in relation to plant nutrition. Soil Sci., 11:49-65.
53. TRAUTWEIN, K.  
1921. Beitrag zur Physiologie und Morphologie der Thionsäurebakterien (Omelianski). Centbl. Bakt. II., 53:514-548.
54. WAKSMAN, S. A.  
1922. Microorganisms concerned in the oxidation of sulfur in the soil. Jour. Bact., 7:231-238.
55. WAKSMAN, S. A.  
1922. Microorganisms concerned in the oxidation of sulfur in the soil: III. Media used for isolation of sulfur bacteria from the soil. Soil Sci., 13:329-336.
56. WAKSMAN, S. A.  
1922. Microorganisms concerned in the oxidation of sulfur in the soil: IV. A solid medium for the isolation and cultivation of *Thiobacillus thiooxidans*. Jour. Bact., 7:605-608.
57. WAKSMAN, S. A.  
1922. Microorganisms concerned in the oxidation of sulfur in the soil: V. Bacteria oxidizing sulfur under acid and alkaline conditions. Jour. Bact., 7:609-616.
58. WAKSMAN, S. A., AND J. S. JOFFE  
1921. Acid production by a new sulfur oxidizing bacterium. Science, 53:216.
59. WAKSMAN, S. A., AND J. S. JOFFE  
1922. Microorganisms concerned in the oxidation of sulfur in the soil: II. *Thiobacillus thiooxidans*, isolated from the soil. Jour. Bact., 7:239-256.
60. WAKSMAN, S. A., CLARA H. WARK, J. S. JOFFE AND R. L. STARKEY  
1923. Oxidation of sulfur by microorganisms in black alkali soil. Jour. Agr. Res., 24:297-305.
61. WINOGRADSKY, S.  
1888. Beiträge zur Morphologie und Physiologie der Baeterien. I. Zur Morphologie und Physiologie der Schwefelbakterien. Leipzig; Felix.





# OBSERVATIONS ON BACILLUS CALIDOLACTIS<sup>1</sup>

R. V. HUSSONG AND B. W. HAMMER

*From the Laboratory of Dairy Bacteriology, Iowa Agricultural Experiment Station*

Accepted for publication September 14, 1931

*Bacillus calidolactis* was originally isolated at the Iowa Agricultural Experiment Station (3) from skimmilk supplied by a milk plant in the state. This plant was pasteurizing surplus skimmilk at 71° to 77° C. and holding it hot in a wooden tank until it could be handled by the drying equipment; occasionally, milk in the wooden tank coagulated and since the temperature in the tank fell slowly, because of the large volumes of milk and the slow heat transfer, the coagulation occurred when the milk was still at a comparatively high temperature. *B. calidolactis* was isolated from pasteurized skimmilk taken from the tank while still in a normal condition and shipped in ice to the laboratory. Milk was transferred aseptically from the shipping containers to sterile test tubes and held in an incubator at 71° C. Coagulation occurred in about eighteen hours and the responsible organism was readily isolated by smearing the milk on slopes of beef extract agar containing one per cent glucose.

The original description of *B. calidolactis* was published as a result of the study of cultures from only one source because it appeared that the organism might be of considerable importance in dairy products, and accordingly an immediate description was desirable. In an investigation of thermophilic and thermoduric organisms from various sources, Prickett (4) secured a number of cultures from pasteurized milk and from milk powder that he identified as *B. calidolactis*.

Cultures of *B. calidolactis* were recently secured from two additional sources, and were studied in order to (a) check and enlarge the description and (b) secure additional information on the changes produced in milk. Comparisons were made with two cultures representing the original isolation of *B. calidolactis*.

## SOURCES OF ORGANISMS

*Source 1.* A number of cans of evaporated milk were received from a condensery having trouble with a peculiar type of coagulation. Three cans that showed little evidence of coagulation on shaking were held at 50° C. and two of them coagulated in a few days while the third did not. Both cans of coagulated milk readily yielded an organism, in what appeared to be a pure culture, when small amounts of milk were transferred to beef infusion agar slopes and the slopes held at 50° C. The organisms from the two cans seemed to be identical. They rapidly coagulated tubes of litmus milk at 50° C., but failed to grow at 37° C., either on beef infusion agar slopes or in litmus milk. Because of their thermophilic character, it was evident the organisms were unrelated to the difficulty experienced at the condensery. A culture from each can was studied.

<sup>1</sup>General Paper No. B3 of the Iowa Agricultural Experiment Station.

*Source 2.* Cans of coagulated evaporated milk were received from a condensery that reported rapid coagulation of cans of milk held at 54.4° C., while no such change occurred in cans held at 37° C. Organisms were easily isolated from the coagulated material when it was cultured on beef infusion agar slopes and the slopes incubated at 55° C. Litmus milk inoculated with the coagulated material rapidly coagulated when incubated at 55° C. There was no growth either on agar slopes or in litmus milk cultures incubated at 37° C. The cultures studied came from four cans of coagulated milk representing the output of four days; they appeared to be identical.

## ACTION OF *B. CALIDOLACTIS* IN MILK

### GENERAL ACTION

*B. calidolactis* rapidly coagulates milk at 55° C. through the formation of acid; tubes of milk inoculated in the usual way are commonly curdled in from sixteen to twenty hours, although longer periods are occasionally required. When litmus is present it is quite rapidly reduced except in the top portion, where a pink layer remains; at 55° C. the color rapidly returns to the reduced portion. Ordinarily there is no evidence of digestion, although occasionally a culture shows some whey. The results of the amino and soluble nitrogen determination indicate that a slight proteolysis occurs.

The general type of change produced by *B. calidolactis* in litmus milk at 55° C. closely resembles that produced by *Streptococcus lactis* or *Lactobacillus bulgaricus* at a somewhat lower temperature.

### TOTAL ACID PRODUCTION

The total acid produced in skimmilk was determined by titrating cultures grown for three days at 55° C., using phenolphthalein as an indicator, and calculating as lactic acid. The acidities found ranged from 0.47 to 0.53 per cent. The skimmilk was firmly curdled in all cases; the firm curd at the low acidities was presumably caused by the combined effect of acid and high temperature.

### TYPE OF NON-VOLATILE ACID PRODUCED

The type of non-volatile acid produced in milk by each of the strains was investigated by preparing zinc salts, using the method outlined by Hammer (1), and examining these. The percentages of ZnO in the salts agreed with the theoretical for zinc lactate and the moisture values indicated that the lactates were entirely or very largely the salts of active acid. The 1 rotation of the salts showed the acids were of the *d* type. The general results indicate that *B. calidolactis* produces *d* lactic acid and this is in agreement with the results presented in the original description of *B. calidolactis* (3).

### VOLATILE ACID PRODUCTION

The volatile acid production in skimmilk cultures grown for three days at 55° C. was studied with the procedure used by Hammer and Bailey (2); this consists of distilling a 250-gram portion with steam after the addition of 15 ml. of approximately  $n/1$   $H_2SO_4$ , and expressing the volatile acidity as the number of milliliters of  $n/10$  NaOH required to neutralize the first

1,000 ml. of distillate, using phenolphthalein as an indicator. The volatile acidities found ranged from 6.0 to 10.0 and showed that *B. calidolactis* produces comparatively little volatile acid. No attempt was made to determine the type of volatile acid because of the small amount formed.

#### ACTION ON NITROGENOUS CONSTITUENTS OF MILK

The action on the nitrogenous constituents of milk was determined as follows: Skimmilk in 200 ml. quantities was sterilized in pint milk bottles and after cooling was inoculated with the strains to be studied. After incubating for one week at 55° C. the bottles of milk were made up to weight (as determined before sterilizing) by the addition of distilled water. One ml. of glacial acetic acid was then added to each bottle and the bottles were heated to 60° C., with frequent shaking, cooled and the contents filtered through paper. Total amounts of soluble nitrogen and amino nitrogen were determined on the whey. In general, there were small increases in both the total soluble nitrogen and the amino nitrogen in the skimmilk in which the organism had grown, but these increases varied considerably with the different cultures.

#### ACTION OF *B. CALIDOLACTIS* IN EVAPORATED MILK

Since *B. calidolactis* was found in evaporated milk from different condenseries, the general action of the organism on evaporated milk was studied. The various strains were inoculated into normal cans by covering a small area on the end of a can with conc. HCl, flooding the area with solder, punching a hole in the center of the area with a nail which had been heated to redness and cooled and then adding the culture; after inoculation the can was immediately resealed with solder and shaken thoroughly to distribute the organisms.

#### GENERAL ACTION

The cultures inoculated into cans of evaporated milk regularly brought about rapid coagulation at 55° C., but at 37° C. no change occurred. The coagulated milk had a distinctly acid and slightly cheesy odor and flavor.

#### NUMBERS OF ORGANISMS IN COAGULATED EVAPORATED MILK

In one trial in which the inoculations of cans of evaporated milk were made in duplicate, examinations were carried out after two days and after five days at 55° C. for the general numbers of organisms present. Because of the difficulty with which the organisms developed on agar and the rapid drying of plates at 55° C., the various dilutions were inoculated into tubes of litmus milk rather than into plates. With this procedure the presence of the organisms could be detected after incubation and the general numbers present per milliliter calculated. The data showed that with two days' incubation *B. calidolactis* was usually present in 1-100,000 ml. but not in 1-1,000,000 ml., while after five days' incubation it was usually present in 1-10,000 ml. but not in 1-100,000 ml. These results suggest that the organisms are not present in large numbers in evaporated milk incubated at 55° C. and that a decrease soon begins. Microscopic examinations also showed that the numbers of organisms present were not large.

## TOTAL ACID PRODUCTION

A number of the cans of inoculated evaporated milk which had been incubated seven days at 55° C. were titrated for total acid, using phenolphthalein as an indicator and calculating as lactic acid. The acidities found varied from 0.67 to 0.74 per cent. Both the normal and the curdled milk were brown and titrations were difficult.

DESCRIPTION OF *B. CALIDOLACTIS*

## MORPHOLOGY (Cultures grown at 55°C.)

Form and size—Rods; 0.7 to 1.4 by 4.2 to 7.0 microns when grown about 18 hours in litmus milk.

Arrangement—Singly and in pairs when grown in milk.

Motility—Motility was not observed and flagella could not be demonstrated.

Staining—The cells stained readily and were gram positive in young cultures, while in old cultures many cells were gram negative.

Spore formation—Oval, terminal spores were present in milk cultures after 18 hours incubation; the cells were swollen by the spores. Ordinarily spores were numerous in milk cultures incubated 18 hours or more.

## CULTURAL CHARACTERISTICS (Cultures grown at 55°C.)

Agar slope—Growth on standard agar was very scanty and with some cultures was absent. On standard agar containing glucose small, round, flat, grey, glistening colonies developed. The edges varied with different strains from smooth to curled.

Agar stab—In standard agar stabs growth was questionable or extremely scanty, while in standard agar containing glucose a very scanty beaded growth developed.

Gelatin—Gelatin was not liquefied.

Potato—No growth.

Carrot—No growth.

Litmus milk—The action in litmus milk was quite characteristic. The milk was coagulated and the litmus was reduced except for a pink band at the surface. Occasionally some whey was evident.

Bouillon—Growth was scanty or absent in plain bouillon and in bouillons containing substances which the strains were not capable of fermenting. When fermentable substances were present there was a faint turbidity which quickly cleared, leaving a slight sediment.

## BIOCHEMICAL FEATURES (Cultures grown at 55° C.)

Gas formation—Gas formation was not observed.

Indol—Indol was not formed in tryptophane broth; a few of the strains would not grow in this medium.



Ammonia—Ammonia was formed.

Nitrates—Some strains reduced nitrates to nitrites in 24 hours, while others did not, even with extended incubation.

Acetylmethylcarbinol from glucose—Acetylmethylcarbinol was not formed from glucose.

Fermenting power—All the strains formed acid but no gas from galactose, glucose, levulose, lactose, maltose, raffinose and dextrin, and failed to ferment adonitol, inositol, dulcitol, mannitol, sorbitol and inulin; the production of acid from glycerol, sucrose, salicin and starch was variable with the different strains.

#### GROWTH CONDITIONS

Oxygen relationship—Facultative; grew well aerobically.

Temperature relationship—Growth was rapid at from 55° to 60° C.; no growth was observed at 37° C. or lower.

#### LITERATURE CITED

1. HAMMER, B. W.  
1920. The type of lactic acid produced by starters and by organisms isolated from them. Iowa Agr. Exp. Sta. Res. Bull. 65.
2. HAMMER, B. W., AND D. E. BAILEY  
1919. The volatile acid production of starters and of organisms isolated from them. Iowa Agr. Exp. Sta. Resh. Bull. 55.
3. HUSSONG, R. V., AND B. W. HAMMER  
1928. A thermophile coagulating milk under practical conditions. Jour. Bact., 15:179.
4. PRICKETT, P. S.  
1928. Thermophilic and thermoduric microorganisms with special reference to species isolated from milk. V. Description of spore-forming types. N. Y. (Geneva) Agr. Exp. Sta. Tech. Bull. 147.



## THE REACTION OF CUCUMBERS TO TYPES OF MOSAIC<sup>1</sup>

R. H. PORTER<sup>2</sup>

From the Plant Pathological Laboratory, Department of Botany, Iowa State College

Accepted for publication September 25, 1931

Cucumber mosaic was probably recognized as early as 1902, but it was not until 1916 that Doolittle (9) and Jagger (25), working independently, established definite proof that it was a transmissible virus disease. Since that time, mosaic has come to be regarded as one of the most destructive diseases of the cucumber. The host range, overwintering hosts and methods of dissemination of the disease, have been determined by Doolittle and Gilbert (10), Doolittle (11, 12) and Doolittle and Walker (14). Their investigations resulted in efforts to control mosaic, by field roguing of wild hosts, and spraying to control insect vectors. Unfortunately these methods have failed in actual practice and mosaic has continued to be one of the primary limiting factors in cucumber production in the United States.

The problem of control becomes complex because of the ease with which many plant viruses are transmitted, and the ability of certain viruses to live on unrelated perennial hosts. Obviously, the discovery or production of a crop variety resistant to a specific virus offers the most satisfactory control measure.

Reddick and Stewart (40) found that the bean varieties White Marrow and Robust were highly resistant to bean mosaic. Folsom (22) stated that the potato variety Irish Cobbler remained free from mosaic in contrast to the varieties Green Mountain and Bliss Triumph, and Murphy (33) found eight varieties of potatoes in Ireland that were resistant to mosaic. Brandes (4) was the first to find mosaic on sugar cane in the United States. He isolated a number of varieties which remained free from mosaic in the midst of heavily infested areas. In 1924, Brandes (5) classified varieties of sugar cane as immune, resistant and tolerant. Edgerton (15) isolated a resistant variety "L 511" by selection and propagation of disease free plants, and Rands and Sherwood (39) found that they could isolate resistant strains of sugar cane by mass selection. Carsner (8) in 1926 found that one strain of sugar beets was considerably less injured by curly top than any other he used and he concluded that the development of a highly resistant strain of sugar beets was feasible. Smith (42) succeeded in hybridizing a variety of Manchuria spinach highly resistant to mosaic with the variety Savoy and produced the resistant variety called Virginia Savoy.

In the summer of 1925 and again in 1926 the writer, while engaged in phytopathological work in China, observed that the cucumber variety

---

<sup>1</sup>A thesis submitted to the Graduate Faculty of Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>2</sup>The writer wishes to express his appreciation to Dr. I. E. Melhus, under whose direction the study was carried on, for his helpful advice and criticism, to Dr. J. C. Gilman for his critical reading of the manuscript, to Dr. E. W. Lindstrom for helpful suggestions on the breeding aspects and finally to Messrs. E. M. Summers, John Leedy, M. H. Berberian and M. E. Yount for aid in growing the plants and in making some of the inoculations.

Chinese Long was free from mosaic. This condition seemed significant, since several varieties of pumpkins and squashes growing in the same gardens with cucumbers were commonly infected. Seed of this variety of cucumbers was sent to Dr. O. H. Elmer at Ames, Iowa, in 1926. He grew plants, inoculated them with cucumber mosaic virus, and the following year reported (20) that they were resistant to the disease. In the spring of 1927, the writer returned to Iowa State College and began a further study of the reaction of this variety to the mosaic disease.

This investigation includes a report of (1) the expression of the "white pickle" virus in the cucumber variety Chinese Long and in inbred lines within this variety; (2) the existence of types of cucumber mosaic; and (3) the reaction to the "white pickle" virus of the progenies from cucumber hybrids involving resistant and susceptible parent stocks.

#### METHODS

All of the plants used for study were grown in the greenhouse, first in two-inch pots, later transferred to four- or five-inch pots and again to six- or eight-inch pots, depending on the length of time the plants were to be kept. In some cases the plants were transplanted into greenhouse benches and kept until the fruits were ripe. An effort was made to use for experiment only those plants which were in a vigorous growing condition. This is especially important when working with cucumbers because few plants are more sensitive to environmental conditions. Extremes of temperature, moisture or light will affect the plants so adversely that symptoms of mosaic may be masked or more commonly the plants will not become infected. The plants were kept in one of two rooms in the greenhouse with air temperatures between 75° and 85°F., except in the summer months, and although they were not covered to prevent insect visitation, an effort was made to keep them free from insects by fumigating frequently and by spraying when necessary. It is significant that in the four-year period covered by these studies, chance infection was seldom observed.

The plants were not given the first inoculation until at least the third or fourth leaf above the cotyledons had appeared. Subsequent inoculations were made as desired. The method of inoculation most commonly used was the one described by Elmer (19) in which strips of paper toweling were employed to hold the leaf receiving the inoculum so that the hands never touched the plants. The leaves were punctured with a sterile needle through a drop of the inoculum prepared by grinding diseased tissue mixed with quartz sand in a sterile mortar. Either sterile or tap water was used to mix with the macerated tissue. Punctures were made in two places: (1) at the base of the leaf near the petiole attachment, and (2) in the petiole. From 15 to 25 stabs were made at each locus. In some cases sterile cheese cloth dipped in the virus culture was rubbed lightly over the leaves.

In all tests to determine the resistance of cucumbers to mosaic, the "white pickle mosaic virus" was used as the inoculum. This virus has been named (*Cucumber virus 1*) by Johnson (29). For most of the tests in 1927, the source of the inoculum was a columbine plant (*Aquilegia canadensis* L.) supplied by Elmer. In October this plant died because of a root rot and it was necessary to use the virus collected during the summer on *Asclepias syriaca* L. near Conesville, Iowa, as the source of the inoculum.



This virus was used until December, 1928, when greenhouse studies were suspended until the following spring. In May, 1929, more plants of *Asclepias syriaca* infected with mosaic were found at Ames, Iowa, and these furnished the source of the inoculum in all subsequent studies unless otherwise stated.

Most of the inoculation trials were made on cucumber plants of the varieties Arlington Improved White Spine, Chicago Special and Chinese Long. In addition, a number of selfed lines were produced and their reaction to mosaic was tested along with plants from open-pollinated fruits. The terms WS-1 and CL-1 were used to designate inbred lines within the varieties White Spine and Chinese Long, respectively.

#### COMPARATIVE RESISTANCE OF THE VARIETIES CHINESE LONG, WHITE SPINE AND CHICAGO SPECIAL TO "WHITE PICKLE MOSAIC"

Elmer (20) in 1926 conducted the first inoculation trials with plants of the variety Chinese Long. He inoculated 50 plants each of the Chinese Long and White Spine varieties with the virus of "white pickle mosaic." None of the former but all of the latter plants became infected with mosaic. In the field trials Elmer planted five six-rod rows of the Chinese Long and transferred aphids to them from mosaic cantaloupe plants, but secured no infection.

On July 11, 1927, the writer inoculated 140 Chinese Long plants and 42 White Spine plants with mosaic virus from *Aquilegia canadensis*. On July 21, all of these plants were transplanted to the field and observed during the summer. Thirty of the White Spine plants showed mosaic, but none of the Chinese Long plants developed any symptoms. Two and one-half rows, each sixteen rods long, of Chinese Long plants were carefully observed until frost in the fall. None of the plants developed any symptoms of mosaic, whereas, several rows of White Spine plants in the same field showed an incidence of at least 50 per cent infection. On October 15, inoculations were made into 22 Chinese Long plants, but none developed mosaic. An additional 150 Chinese Long plants, 10 Crookneck squash plants and 4 White Spine plants were inoculated with mosaic virus on October 29. By November 14 two of the squash plants had developed mosaic, but all of the Chinese Long and White Spine plants remained free. On December 14, 25 Chinese Long plants were inoculated with virus from a squash plant which had been inoculated from a mosaic milkweed. Four squash plants were inoculated as checks and two of them became infected. All of the Chinese Long plants remained healthy.

The inoculation trials with Chinese Long plants during 1926 and 1927 gave rather strong indications that this variety was resistant to mosaic. However, the number of tests made had not been sufficient to establish this fact completely. Furthermore, the possibility of masking in this variety was considered an important question and attempts were made to answer it.

#### EXPERIMENTS IN 1928

The results of all inoculation trials in 1928 with the variety Chinese Long were summarized and are presented in table 1. The tests included not only plants grown from seed of open pollinated fruits, but also plants from fruits in which self-fertilization had been practiced for one or two generations. These experiments disclosed the fact that an occasional Chinese Long

TABLE 1. *Reaction of cucumber plants to the virus of "white pickle mosaic" (Cucumber virus 1) 1928*

Source of virus	Plants inoculated	Date	Date symptoms appeared	Total plants inoculated	Total plants infected
Crookneck squash	Chinese Long	Mar. 2	Not recorded	20	1
"	X25 <sup>1</sup>	" 2	"	29	8
"	Chinese Long	" 9	—	15	0
"	X25 <sup>1</sup>	" 9	Not recorded	4	2
"	Crookneck squash	" 9	"	3	1
—	Ten checks	" 9	"	0	0
Crookneck squash	Chinese Long	" 19	Mar. 26	25	5
"	Crookneck squash	" 19	" 23	6	1
"	Chicago Special	" 19	" 23	5	4
"	Chinese Long	Apr. 4	Not recorded	11	2
"	Chicago Special	" 4	"	21	16
"	X25 <sup>1</sup>	" 4	"	20	10
"	Chinese Long	" 5	—	10	0
"	Chicago Special	" 5	Not recorded	10	6
"	Crookneck squash	" 5	—	10	0
—	Ten checks	" 5	—	0	0
Crookneck squash	Chinese Long	" 21	—	120	0
"	Chicago Special	" 21	Not recorded	20	7
"	Chinese Long	" 27	May 15	40	1
"	Chicago Special	" 4	" 5	13	9
White Spine	CL11-1	June 23	July 7	7	2
"	CL4-1	" 23	" 7	8	4
"	Chinese Long	" 23	" 10	30	6
"	White Spine	" 23	" 1	26	15
—	Ten checks	" 23	—	0	1
White Spine	Chinese Long	July 30	—	9	0
"	Chicago Special	" 30	Aug. 6	10	6
"	Chinese Long	" 30	" 12	20	5
"	White Spine	" 30	" 8	20	15
"	Chinese Long	Aug. 20	—	10	0
"	Chicago Special	" 20	Aug. 28	10	8
"	CL8-1	" 6	—	10	0
"	CL2-2	" 6	Aug. 12	2 <sup>2</sup>	2
"	CL1-1	" 6	" 12	15	2
"	CL11-1	" 6	" 28	6	2
"	CL20-2	" 6	—	10	0
"	CL6-1-2	" 6	—	10	0
"	CL2-1-4	" 6	Aug. 28	10 <sup>3</sup>	2
"	CL41	" 6	" 28	10	6
"	CL6-1	" 6	" 18	10	1
"	CL5-1	" 6	" 15	10	5
"	Chicago Special	" 6	" 12	5 <sup>4</sup>	4
Ten checks		" 6	—	0	0

<sup>1</sup>F<sub>1</sub> from WS x CL.<sup>2</sup>Plants died apparently from mosaic.<sup>3</sup>One plant showed symptoms Aug. 16.<sup>4</sup>Two plants showed symptoms Aug. 12.

plant was susceptible to mosaic. The first instance of this condition was discovered in March, 1928. On March 2, 20 Chinese Long plants and 29 F<sub>1</sub> plants resulting from a cross of Chinese Long x White Spine were inoculated with mosaic. Eight of the F<sub>1</sub> plants developed mosaic and one Chinese

Long plant showed a slight mottling resembling mosaic. This plant was saved for further study. On March 19, inoculations were made to 25 Chinese Long plants. On March 25, one of these plants developed mottling typical of mosaic and four more showed attenuated symptoms the following day. Of the checks, six out of eleven developed mosaic four days following the inoculation.

The next instance of mosaic symptoms on the variety Chinese Long occurred following the inoculation on April 4, of 11 plants, together with 21 Chicago Special and 20 F<sub>1</sub> plants. Sixteen of the Chicago Special and 10 of the F<sub>1</sub> plants developed mosaic and two Chinese Long plants were suspected, but in a few days they recovered, as evidenced by normal growth of healthy leaves. On April 27, 40 Chinese Long plants were inoculated and one showed typical mottling on May 15. This made a total of nine plants of this variety which had showed faint or prominent mosaic symptoms, but this condition was detected only after more than 800 plants had been subjected to mosaic by artificial inoculation. A further discussion of this condition will be taken up later.

Additional tests during the summer and fall included selfed lines and plants from open pollinated fruits of the variety Chinese Long. In most cases, as shown by table 1, this variety continued to exhibit a high degree of resistance to mosaic.

A summary of the results secured in 1928 show that out of a total of 494 plants, representing mass selected and self-fertilized fruits of the variety Chinese Long, 46 became infected with mosaic. This is equal to 9.3 per cent. At the same time, a total of 209 plants, representing susceptible varieties, were inoculated and 112 or 53.6 per cent became infected with mosaic.

Inbreeding within the variety Chinese Long resulted in the production of three lines: CL6-1-2, CL8-1 and CL20-2, which remained free from mosaic, but the same process produced several lines that were moderately susceptible. Line CL4-1 in two tests showed 55.5 per cent, CL5-1 showed 50 per cent, and CL11-1 showed 30.8 per cent mosaic-infected plants. The response of these plants was such that the term, tolerant, is more descriptive than susceptible. In a later section it will be shown that this peculiar response was typical of Chinese Long plants which developed symptoms of mosaic.

#### RESULTS OF TESTS IN 1929

As stated earlier in this paper, the source of the mosaic virus used in 1929 was a mosaic milkweed plant, *Asclepias syriaca*, found in the plots at Ames, Iowa. The results of all of the tests in 1929 are presented in table 2, in which inbred lines as well as open pollinated strains were subjected to inoculation with the cucumber mosaic virus. The data in tables 1 and 2 show that some of the selfed lines of the variety Chinese Long did not react in the same way to the virus used in 1929 as they did in 1928. For example, in 1928 lines CL11, CL4, CL2, CL1 and CL5 all were susceptible, whereas in 1929 the first four lines remained free from mosaic following inoculation. Line CL5 was not included in the 1929 test. One line, CL16-1, showed susceptibility in 1929, three out of 16 plants developing slight symptoms, although even in this case there was some question that the effect was caused by mosaic. One out of ten Chinese Long plants inoculated

on June 22 developed typical mosaic symptoms July 3. The results of the tests made in 1929 show that out of 150 plants of the variety Chinese Long, representing seven inbred lines and three open pollinated lots, four became infected with mosaic. In contrast to this, 36 plants of susceptible strains were inoculated and 29 or 80.6 per cent of them became infected.

TABLE 2. *Reaction of cucumber plants to the virus of "white pickle mosaic" (Cucumber virus 1) 1929*

Source of virus	Plants inoculated	Date	Date symptoms appeared	Total plants inoculated	Total plants infected
Milkweed	Chinese Long	June 22	July 3	10	1
"	WS7-1-1	" 22	June 26	5	5
"	CL11-1-1	" 22	—	4	0
"	X25 <sup>1</sup>	" 22	July 3	10 <sup>2</sup>	10
White Spine	CL20-1-2	" 25	—	12	0
	Ten checks	" 25	—	0	0
White Spine	CL2-2-1	" 25	—	19	0
"	CL4-1-1	" 25	—	16	0
"	CL1-1-2	" 25	—	22	0
"	Chinese Long	" 25	—	3	0
"	CL2-2-1-1	" 25	—	2	0
"	CL8-1-1	" 25	—	12	0
"	CL11-1-1	" 25	—	10	0
"	White Spine	" 22	July 3	10 <sup>3</sup>	5
"	CL16-1	" 25	" 8	16	3
"	CL16-1	" 29	—	6	0
"	White Spine	" 29	July 3	6	6
"	CL4-1-1	" 29	—	3	0
"	WS7-1-1	Sept. 3	Sept. 16	5 <sup>4</sup>	4
"	Chinese Long	" 3	—	5	0
"	CL8-1-1	" 3	—	5	0
"	WS5-1-2	" 3	Sept. 16	5	1
"	White Spine	" 3	" 16	5	3
"	CL4-1-1	" 3	—	5	0
"	Five checks	" 3	—	0	0

<sup>1</sup>F<sub>1</sub> hybrid of CL x WS.

<sup>2</sup>Six plants showed symptoms June 26 and two June 29.

<sup>3</sup>Two plants showed symptoms June 29.

<sup>4</sup>Three plants showed symptoms Sept. 9.

#### CROSS INOCULATION FROM PLANTS OF THE VARIETY CHINESE LONG ON SUSCEPTIBLE CUCUMBERS

The development of mosaic symptoms on an occasional plant of the variety Chinese Long suggested the possibility of the symptoms being masked. Five plants which had been inoculated on March 19, 1928, showed mosaic symptoms on March 29. From each of these, cross inoculations were made to five plants of the variety Chicago Special. In two cases, two plants out of the five developed mosaic with attenuated symptoms. From two of these infected plants, transfers were made on April 21 to nine Chicago Special plants, of which six developed typical mosaic. During the summer of 1928, additional experiments were conducted, the results of which are



presented in table 3. It is evident from the results in this table that cucumber plants of the variety Chinese Long which developed mosaic symptoms carried the virus. A total of 17 plants in these tests showed faint to prominent mottling and transfers were successfully made from 12 of them to cucumbers known to be susceptible. Unsuccessful transfers may have been partially due to poor growth of the plants subsequent to the inoculation.

TABLE 3. *Cross inoculations to known susceptible hosts from Chinese Long cucumber plants which had developed mosaic symptoms, 1928*

From	Transfers To	Number inoculated	Date first inoculated	Date first symptoms appeared	Total plants inoculated	Total plants infected
CL4-2	Chicago Special	5	July 9	July 13	5	2
CL11-2	"	5	" 9	" 13	5	2
CL11-2-1	"	5	" 19	Aug. 6	5	3
CL4-1	"	5	" 13	—	5	— <sup>1</sup>
CL11-1	White Spine	5	" 13	—	5	— <sup>2</sup>
CL33	Chicago Special	3	Aug. 16	Aug. 28	3	3
CL33	"	3	" 23	" 28	3	3
CL34	"	3	" 14	" 28	3	3
CL35	"	3	" 14	" 28	3	1
CL35	"	3	" 23	Sept. 12	3	3
CL36	"	3	" 14	" 28	3	2
CL37	"	3	" 14	Aug. 28	3	2
—	Ten checks	—	—	—	0	0

<sup>1</sup>Plants grew poorly.

<sup>2</sup>CL11-1 did not show distinct mottling.

While evidence was being accumulated to show that plants which developed mosaic symptoms were actually infected with the mosaic virus, it seemed advisable to learn whether inoculated plants which, to all outward appearance were free, in reality were carriers of mosaic. Nishimura (34) has shown that *Physalis alkekengi* L. may carry the virus of tobacco mosaic without showing any symptoms, and Melhus (31) found that egg plant (*Solanum melongena* L.) recovered from mosaic symptoms after the plants had passed the seedling stage. Brierly (6) and Verwoerd (43) found that tomato plants recovered from mosaic.

In 1928, 1929 and 1930 series of tests were conducted, using as the source of the inoculum Chinese Long plants, which, following inoculation, showed no symptoms. In the first trials 13 plants were used and from only one was the mosaic disease successfully transferred. In May, 1930, 20 plants consisting of ten each from the lines CL8-1-1 and CL11-1 were planted in eight-inch pots and inoculated three times with mosaic virus at intervals of one week. After the third inoculation, transfers were made from each one of them to two plants of the variety White Spine. From two plants of CL8-1-1 and three of CL11-1 mosaic was successfully transferred, although no symptoms were observed on any of them. These plants may be considered as mosaic carriers. The remainder of the set were apparently so highly resistant that the virus was unable to establish itself. These re-

sistant plants have been further inbred and their progenies used in hybridization trials with plants of susceptible varieties.

GROWTH RESPONSE TO MOSAIC OF THE VARIETIES CHINESE LONG, WHITE SPINE  
AND CHICAGO SPECIAL

From the beginning of the work with the variety Chinese Long, it became evident that its general vigor was somewhat greater than the American sorts we had been using. In the greenhouse, under the same conditions, Chinese Long plants made more rapid gains in size of plants and fruits and in number of leaves than the White Spine plants. Under field conditions, Chinese Long plants usually blossomed earlier and produced more fruits per plant than did either White Spine or Chicago Special plants.

During the summer of 1928, after it was discovered that an occasional Chinese Long plant developed mosaic, it became apparent that the symptoms of mosaic in this variety were somewhat different than on common American sorts. One symptom was a faint mottling of the terminal leaf with more yellow spots than commonly occur on White Spine plants. Mottling was usually observed only on the terminal leaves. In one or two instances, plants showed no mottling, but the upper leaves were decidedly crinkled. Transfers from these plants to susceptible ones proved the presence of the mosaic virus. One typical symptom of mosaic, which was so common in all American varieties but entirely lacking in Chinese Long in all tests to date, was the "white pickle" or warty fruits. This reaction gives further evidence that the behavior of the variety Chinese Long to mosaic is strikingly different than that of any other variety thus far studied.

A second point of difference in response of this resistant variety was the lack of marked stunting. In the greenhouse, one of the earliest noticeable symptoms of mosaic in the susceptible varieties was pronounced stunting, accompanied by drooping or downward curling of the juvenile leaves. Occasionally a plant recovered somewhat from this depression, but such recovery was rare. In the variety Chinese Long, a plant which developed mottling showed little or no stunting; in fact, within a few days or a week there seemed to be no apparent difference in the vegetative vigor of tolerant and resistant plants. It was also common for infected plants of this variety to recover within a few days so that no macroscopic symptoms were detectable. This reaction is well illustrated by an experiment conducted in 1928. On July 30, 20 Chinese Long plants were inoculated with mosaic, and on August 12, five showed faint mottling of the terminal leaves. On August 15 these symptoms had entirely disappeared and if they had not been noted, it would have been impossible to have selected the ones with mosaic from those which were free. These infected plants still retained the virus because successful transfers were made as late as August 23, as shown by CL33 in table 4. In another case, plant CL11-1, which developed mosaic symptoms on July 7, had recovered by July 13. If we consider these recovered plants free from symptoms we must admit that plants of this variety may be carriers of mosaic.

Further tests to determine the relative amounts of vegetative growth of varieties Chinese Long and White Spine following inoculation with mosaic were conducted, using the same plants that are recorded in table 3. On July 30, 1928, 20 Chinese Long plants were inoculated with mosaic and on August 12, five plants were mottled, none were stunted, and they all continued to grow vigorously. The average height on August 28 of all 20

plants was five and one-half feet. All but one of these plants had produced normal fruits. In contrast, 20 White Spine plants were inoculated at the same time and kept under the same conditions. Fifteen of these developed mosaic and the average height of the entire 20 plants was two and one-half feet. Three out of the 20 plants developed normal fruits. On August 20, ten more Chinese Long plants were inoculated with mosaic. They were transplanted to the bench August 23 and closely observed until late in September. None of them developed symptoms of mosaic. The average height was about four feet. At the same time ten plants of the variety Chicago Special were inoculated with mosaic and by August 28, eight of them were severely stunted and mottled. On September 12, every one of the ten plants was dead as a result of mosaic infection.

#### SUMMARY OF THE RESPONSE OF PLANTS OF THE VARIETY CHINESE LONG TO "WHITE PICKLE" MOSAIC

It is evident from the data presented in the foregoing pages that, considered as a variety, the Chinese Long is heterozygous in its reaction to mosaic. A small percentage of plants grown from seed of open-pollinated fruits developed faint mottling of the terminal leaves following inoculation. Inbreeding within this variety produced certain lines which are highly resistant to the extent that no symptoms of mosaic were produced and the virus from such plants was seldom recovered even though three successive inoculations were made. Other lines were produced which are highly susceptible as indicated by mottling of the leaves. In many such instances the symptoms remained for a considerable period of time. A third type of response was secured in which the plants developed faint mottling, but recovered so rapidly that no symptoms were observed later. Such plants may be considered as decidedly tolerant to mosaic.

In no case was any marked stunting observed which could be attributed to the effects of the virus. Plants of the variety Chinese Long even though inoculated several times maintained their vegetative vigor and apparently produced normal fruits. It is considered that the physiological response of this variety to mosaic is markedly different than the response of other cucumber varieties thus far studied.

#### TYPES OF CUCUMBER MOSAIC

In any study of a virus disease of plants, the question of the specificity of the causal entity as well as its host range usually arises, especially if the problem involves the production or isolation of resistant lines or varieties.

Jagger (26), in 1917, working with cucumber mosaic, suggested that two types of mosaic virus produced disease in the cucumber, one of which caused "white pickle," stunting of the plants and mottling of the leaves, whereas the other virus produced only leaf mottling. Later he (27) reported that a mosaic virus occurred on summer crookneck squash and pie pumpkin which could not be transferred to cucumber. Schultz and Folsom (41) described seven distinct virus diseases of potatoes, all of which occurred on the variety Green Mountain. Fernow (21) concluded that the mosaic diseases on 19 different species of plants, 15 of which belonged to the Solanaceae, could be divided into eight different types, four of which went to more than one host. Johnson (29) showed that eight different mo-



saics were present on tobacco and related plants, determined on the basis of their behavior to several factors used as tests. Bennett (3) has described three distinct mosaic diseases of the raspberry and two other diseases of the virus type.

In contrast to the above evidence on the specialization of plant viruses, a few investigators have secured results which indicate that a virus may have a wide host range and in addition produce either unusual symptoms on a particular host or have all signs entirely masked. Elmer (17, 19) was able to inter-transmit mosaic to species in 15 different families and 11 different orders. Doolittle and Walker (14) transmitted the virus of cucumber mosaic to 12 different genera, which included plants of 23 species and 96 horticultural varieties. Carsner (7) was able to modify the virus of curly-top in the sugar beet by passing it through any one of the three hosts: *Chenopodium murale* L., *Rumex crispus* L., or *Suaeda moquini* Greene, to the extent that mild symptoms were produced when transferred back to sugar beets. Walker (44) was able to modify the specific properties of the mosaic extracts secured from tomato, cucumber or ground cherry by inter-transmission from one to the other, thus suggesting the possibility of a common causal agent. Hoggan (23, 24) has presented evidence that insects have a selective capacity either for a virus or for a host plant. This is of significance because it emphasizes the importance of various methods of inoculation in order to determine the transmissibility of a particular virus. Mogendorf (32) was able to produce "fern-leaf" of tomatoes regularly with the virus of cucumber mosaic when the peach aphid *Myzus persicae* was used as the vector.

In 1928 and 1929 an effort was made to secure host plants infected with cucumber mosaic from several localities. Samples were secured as follows:

Host plant	Place	Courtesy of
Squash	Yonkers, N. Y.	F. O. Holmes
Cucumber	Manhattan, Kan.	O. H. Elmer
Cucumber	Chula Vista, Calif.	I. C. Jagger
Cantaloupe	Conesville, Iowa	D. R. Porter and D. V. Layton
Cucumber	Davenport, Iowa	Davis Greenhouses
Turkish Tobacco	Lexington, Ky.	E. M. Johnson
Milkweed ( <i>Asclepias syriaca</i> )	Conesville, Iowa	D. R. Porter and D. V. Layton

In addition to the above, samples of mosaic were collected at Ames, Iowa, on milkweed (*Asclepias syriaca*), cucumber, cantaloupe, crookneck squash and nutmeg squash (*Cucurbita pepo*). Inoculation trials with expressed juice from the above hosts to cucumber plants (White Spine variety) produced symptoms similar to the ordinary "white pickle mosaic" disease with two exceptions, namely, nutmeg squash and cucumber plants from the Davis Greenhouses, Bettendorf, Iowa. Three attempts in 1929 to produce infection in variety White Spine cucumber plants with juice from mosaic nutmeg squash plants failed. When the mosaic specimens were collected the host was not known, hence no inoculations were made on the host from which the virus was collected.

The sample of mosaic cucumber plants from the Davis Greenhouses was secured in March, 1929. Correspondence with this company in Janu-



ary, 1929, indicated that mosaic was causing some trouble on their forcing cucumbers. Specimens were sent to Ames, but by the time they arrived the leaves were too dry and no mosaic was secured from them. Later Dr. I. E. Melhus visited the houses at Bettendorf and brought back specimens from which the first successful inoculations were made. Additional specimens secured in March, 1930, by the writer from these greenhouses proved to be infected with the same virus secured the previous year.

Inoculation trials were first made on cucumber plants of the White Spine variety. Each plant became infected, but the symptoms were somewhat different from those produced by the "white pickle virus." Inoculation trials were next made on the plants of the variety Chinese Long and 100 per cent of the plants became infected. Subsequent studies during the summer and autumn (38) with this virus have shown that it is distinct from the "white pickle mosaic virus" described by Doolittle, as shown by differences in period of incubation, symptoms and hosts attacked. This virus will hereafter be referred to as Cucumber virus 2, since Johnson (29) has termed the "white pickle virus" "Cucumber virus 1."

#### EXPERIMENTAL RESULTS WITH CUCUMBER VIRUS 2

The first inoculations with Cucumber virus 2 were made in March, 1929. Subsequent inoculations were made throughout the spring and summer. Table 4 records the cucumber plants subjected to infection with this virus. The inoculum used each time was taken from a young, vigorously growing plant showing typical symptoms of the disease.

The data presented in table 4 show that out of 14 sets of plants which were inoculated, 100 per cent infection was secured in ten of them. Two hundred and nine plants were inoculated and 184 became infected, which is equivalent to 88 per cent. In addition to plants of the varieties White Spine and Chinese Long, some selfed lines within these varieties were used.

The period of incubation for this virus varied with the environmental conditions and with the host used. The minimum period of incubation in all plants of the Chinese Long variety was six days, the maximum 22. The majority of the plants showed symptoms within 15 days or less. In the variety White Spine the minimum period of incubation was five days and the maximum 11 days.

In order to make a fair comparison of the incubation period between the two varieties, it is necessary to refer to the instances where both types were inoculated on the same days with the same inoculum. On June 22, four plants each of Chinese Long and White Spine were inoculated. The former showed symptoms on two plants in 11 days, the latter showed symptoms on two plants in eight days. On June 25, another test was run in which eight plants of CL16 and five of White Spine were inoculated. The first symptoms appeared on CL16 in eight days and on the White Spine in five days. On June 29, in another test, the results were somewhat similar, the first symptoms appearing on CL16 and CL4 in six and nine days, respectively, whereas, on White Spine the first symptoms appeared in five days. It is evident that the period of incubation for Cucumber virus 2 in the variety Chinese Long is one to three days longer than in the variety White Spine.

TABLE 4. *Reaction of cucumber plants to Cucumber virus 2 .*

Source of virus	Plants inoculated	Date inoculated	Date symptoms appeared	Total plants inoculated	Total plants infected
Cucumber from Bettendorf White Spine cucumber	White Spine	Mar. 5	Mar. 13	15	15
	Chinese Long	" 25	Apr. 7		
			" 11		
			" 16	17	17
"	CL4-1	" 25	" 7		
			" 11		
			" 16	21	21
"	CL4-1-2	" 25	" 7		
			" 11		
			" 16	20	20
"	CL8-1-8	May 5	May 21		
			" 23		
			" 24	23	17
"	CL8-1-8	" 24	June 1		
			" 3		
			" 8	71	54
"	X25	June 22	" 30		
			July 1		
			" 3	8	8
"	Chinese Long	" 22	" 3		
			" 6	4	4
"	WS7-1-1	" 22	June 30		
			July 3	4	4
"	CL16-1	" 25	" 3		
			" 4	8	8
"	White Spine	" 25	June 30		
			July 1		
			" 2	5	5
"	CL16-1	" 29	" 5		
			" 6	6	5
"	White Spine	" 29	" 4		
			" 5	6	6
"	CL4-1-2	" 29	" 8	5	2

## COMPARISON OF CUCUMBER VIRUSES 1 AND 2

It has already been stated that Cucumber virus 2 differs from the "white pickle virus" in period of incubation, in symptoms produced and in hosts attacked. Comparisons bearing on the incubation period can only be made on a host susceptible to both viruses and since the Chinese Long cucumber is relatively resistant to Cucumber virus 1, a study was made on plants of the variety White Spine, using both selfed lines and open pollinated material.

Table 5 presents some data indicating that the incubation period for Cucumber virus 2 is two to four days longer than for Cucumber virus 1. The number of days required for the first symptoms to appear when Cucumber virus 1 was used were four, four, four and two, respectively, in the four different sets of inoculations made. The incubation period for Cucumber virus 2 was eight, four and six days, respectively, in the three sets of inoculations made. It is also evident from these data, as well as from many other tests, that a higher percentage of the plants became infected when Cucumber virus 2 was used than with Cucumber virus 1. In table 5 the data show that 100 per cent infection occurred with Cucumber virus 2 and only 73 per cent with Cucumber virus 1. Data from table 4 show that even with a relatively large number of plants, 88 per cent became infected in tests with Cucumber virus 2.

TABLE 5. *Relative periods of incubation for Cucumber viruses 1 and 2*

Virus used	Plants used	Date inoculated	Date symptoms appeared	Total inoculated	plants infected
Cucumber virus 1 From milkweed "	White Spine	June 15	June 19	5	3
	WS7-1-1	" 22	" 26	5	5
	White Spine	" 25	" 29	10	5
			July 1		
			" 3		
Cucumber virus 2 from White Spine "	White Spine	" 29	" 1	6	6
			" 3		
			June 30		
	WS7-1-1	" 22	July 6	4	4
	WS7-1-1	" 25	June 29		
			July 1		
	White Spine	" 29	" 6	5	5
			" 5		
			" 8		

## SYMPTOMS OF CUCUMBER VIRUSES 1 AND 2

One of the most outstanding differences between Cucumber viruses 1 and 2 is the effect produced on hosts of the same strain or variety. The symptoms of Cucumber virus 1 as observed by the writer are similar to those described by Doolittle (11), except that under greenhouse conditions there is little or no mottling produced on young, vigorous plants, which have developed the fourth leaf above the cotyledons at the time of inoculation. These plants show a marked stunting effect two or three days after inoculation. The leaves which receive the inoculum usually droop, curl downward and present a leathery appearance.

Symptoms produced by Cucumber virus 2 in the early stages of infection are distinctly different. The first signs occur either on the terminal leaf or on those receiving the inoculum, in the form of one or more yellow, irregularly shaped spots. Later these spots increase in number and produce a densely mottled pattern of yellow and green as shown in Plate I, figure 1. This condition is similar to the symptoms produced on cucumbers by the ring spot virus except that in the latter case the yellow spots are

smaller and more circular in outline. Symptoms of Cucumber virus 2 and ring spot virus are illustrated in Plate I, figure 1. No stunting effect is manifest with Cucumber virus 2 until a few days after the first symptoms appear. The terminal leaves do not droop, but remain rigid throughout the life of the plant. The writer has never observed any visible symptoms of infection on the fruits.

Plate II, figure 1 shows the response of cucumber plants to Cucumber virus 1. The marked stunting of the White Spine plant on the right is a decided contrast to the Chinese Long plant on the left. The center plant, which is an  $F_1$  hybrid of variety Chinese Long x variety White Spine, shows an intermediate reaction. This plant is X25 recorded in table 4. Plate II, figure 2, is a duplicate of Plate II, figure 1, except that Cucumber virus 2 was used as the inoculum. Each of the plants in these two figures is representative of several plants of the same kind.

#### DIFFERENTIAL HOSTS

Elmer (20 and the writer (35, 36) have referred to the high degree of resistance in cucumber plants of the variety Chinese Long to "white pickle mosaic. Further data have been presented in another chapter of this paper. In contrast to the resistance of this host to Cucumber virus 1, is its striking susceptibility to Cucumber virus 2 as shown in table 4. The Chinese Long cucumber, then, is a differential host for these two viruses.

Another differential host apparently is the watermelon (*Citrullus vulgaris* Schrad.). Thus far, plants of the varieties Tom Watson, Kleckley Sweet, Cuban Queen, Mountain Sweet, Halbert Honey, Thurman Gray and Phinney's Improved have been used in the inoculation tests. Infection does not occur as readily as it does in varieties of cucumber, but mosaic symptoms have been produced experimentally in the greenhouse on plants of all the varieties mentioned above. Some of these plants were later transplanted to the field and the virus again recovered by inoculation into cucumber plants. The symptoms on young plants were faint to prominent mottling of the juvenile leaves, followed by either a slight or severe stunted appearance. Plate III, figure 1, shows the relative size of infected and non-infected watermelon plants. Older plants in the field masked the symptoms, although faint mottling often occurred in the terminal leaves. Plate IV, figure 1, shows both healthy and mosaic leaves taken from Tom Watson plants as a result of infection with Cucumber virus 2. Table 6 presents some data bearing on the susceptibility of watermelon varieties to Cucumber virus 2.

In addition to watermelon as a host, infection with Cucumber virus 2 was secured on plants of the West India gherkin (*Cucumis anguria*), the African citron (*Citrullus vulgaris*) and Green Seeded citron (*Citrullus vulgaris*) as shown in table 7. The symptoms were similar to those on watermelon and are illustrated in Plate III, figure 2, and Plate IV, figure 2. The latter named were also susceptible to Cucumber virus 1. Two plants of each were inoculated on October 6, 1929, and one of each developed distinct mottling on October 11. Stunting was observed and the mottling was more pronounced than when Cucumber virus 2 was used.

The writer has never been able to produce infection in watermelon plants of any variety with Cucumber virus 1. Jagger (27) states that he secured infection on two watermelon plants, variety Cole's Early, with



TABLE 6. *Reaction of watermelon and cucumber plants to Cucurbit virus 2*

Source of virus	Plants inoculated	Date	Date symptoms appeared	Total plants inoculated	Total plants infected
White Spine cucumber (filtrate from fritted glass filter)	White Spine Cucumber	1929 June 13	1929 June 19	5	5
"	Tom Watson watermelon <sup>1</sup>	June 13	June 30	3	2
"	F. (Tom Watson x citron) <sup>2</sup>	June 28	July 10	3	2
Tom Watson watermelon (from greenhouse)	Tom Watson	June 13	July 27	2	2
"	Tom Watson	July 15			
"	White Spine	Aug. 1	Aug. 10	3	3
Tom Watson watermelon (from field)	CL1-1-1 cucumber	Sept. 11	Aug. 8 Sept. 25	3 5	3 5
CL1-1-1-1	Tom Watson	Oct. 6 1930	Oct. 16 1930	2	1
Cucumis anguria carried through the winter)	WS9-1 cucumber	Apr. 10	Apr. 26	3	2
"	WS7-1-2	"	"	2	2
"	X25	"	None	2	0
"	X21	"	Apr. 26	2	2
"	Kleckley Sweet watermelon	"	May 5	5	3
"	Halbert Honey watermelon	"	"	4	3
"	WS9-1	Apr. 11	Apr. 27	2	2
White Spine (virus secured from Davis Greenhouse in March, 1930)	WS7-1-2	"	Apr. 26	2	2
"	X25	"	Apr. 28	1	1
"	X21	"	None	1	0
"	Cuban Queen watermelon	"	May 5	4	2 <sup>3</sup>
"	Thurman Gray	"	"	4	1 <sup>4</sup>
"	Mountain Sweet	"	"	4	3
"	Kleckley Sweet	"	"	4	3
"	Phinney's	"	Apr. 30	4	3

<sup>1</sup>Transplanted to field.<sup>2</sup>Transplanted to field June 28, symptoms appeared on two about July 12.<sup>3</sup>Two plants died early from wilt.<sup>4</sup>Three plants died early.

cucumber mosaic, but he did not prove the presence of the virus by cross inoculation to cucumber. Doolittle and Walker (14) were unable to produce mosaic on Tom Watson, Halbert Honey, Kleckley Sweet, Mammoth Santiago, Olds 1908, and Sweet Heart watermelons. Mosaic in the field has never been observed by the writer on watermelons in Iowa, except on  $F_1$  plants of a watermelon-citron hybrid. In two cases, the virus was recovered from such hybrid plants and transferred to cucumber plants of the White Spine variety, where typical mosaic symptoms of Cucumber virus 1 were produced.

#### EFFECT OF FILTRATION ON CUCUMBER VIRUS 2

Only one attempt has been made to filter the active agent in Cucumber virus 2. In this instance infected leaves from a White Spine cucumber plant were macerated in a mortar, sterile water added, and the mixture was then poured into a fritted glass filtered No. <7. The filtrate was used to inoculate watermelon and cucumber plants as shown in table 6. The filtrate proved highly infectious. At the same time, the residue, to which a small amount of water had been added, was used to inoculate five White Spine plants on June 13. One plant developed mosaic on June 28.

TABLE 7. *Reaction of citron plants to Cucumber virus 2*

Source of virus	Plants inoculated	Date	Date symptoms appeared	Total plants inoculated	Total plants infected
CL1-1-2	African citron	1929 Oct. 6	Oct. 16	5	2
		1930 Apr. 10	May 5	5	3
Cucumis anguria	"	" 30	" 15	8	5
"	Green Seeded citron	" 10	" 5	4	2
"	"	" 30	" 15	8	3
White Spine (virus secured from Davis Greenhouse in March, 1930)	African citron	" 11	" 5	4	2
	Green Seeded citron	" 11	" 15	4	1

#### DISTRIBUTION OF CUCUMBER VIRUS 2

No extensive effort has been made to determine the distribution of Cucumber virus 2. Jagger (26) in 1917 reported a separate and distinct mosaic disease of cucumber near Rochester, New York. He found that it produced a mottling of the leaves, but showed no effect on the fruits. No further description of the symptoms was given, hence it is difficult to make a direct comparison with Cucumber virus 2. Jagger suggested that the disease he worked with might be the same as reported by Selby in Ohio and by Stone in Massachusetts. Cucumber virus 2 has been found in only one locality in Iowa to date, whereas, Cucumber virus 1 is widespread. It is of some significance, however, that mosaic cucumber plants were secured by mail from the Davis Greenhouses in March, 1930, just one year later than

the first sample. Inoculation trials on cucumber, watermelon and citron plants resulted in infection with symptoms identical to those secured the previous year, as shown in tables 6 and 7. The present indication is that the range of Cucumber virus 2 is limited.

#### HYBRIDIZATION EXPERIMENTS WITH CUCUMBERS

##### CLASSIFICATION OF VARIETIES OF CULTIVATED CUCUMBER

The common edible cucumber used in Europe and North America is classified botanically as *Cucumis sativus* L. Bailey (1) states that although there are many varieties of the species in cultivation, in general the group is variable with unstable species and indefinite varietal distinctions. He says, however, that certain types are dominant, that these types may be separated and most of the varieties can be conveniently classified around these types. The principal types, according to Bailey, are as follows:

- I. English forcing type, var *anglicus*. Large leaved, strong-growing, slow-maturing plants; fr. large, long, smooth, usually green with few or early deciduous black spines. Three commercial varieties are given under this type.
- II. Field varieties
  - A. Black Spine.
    1. Netted Russian.
    2. Early Cluster.
    3. Medium Green.
    4. Long Green type. One of the best fixed varieties, representing perhaps one of the most primitive stages in the evolution of the group. Vines large, long and free growing; fr. large and long, green, ripening yellow, with scattered black spines. Long Green and Japanese Climbing are representatives of this type.
  - B. White Spine varieties.
    1. White Spine type.
    2. Giant Peru type. Mostly poorly fixed varieties having large, rather unthrifty vines, bearing large frs. tardily and sparsely, which are white or whitish, smooth or with scattering, deciduous, usually white spines. This type is represented by Chicago Giant, Giant Peru and Long Green.

##### DESCRIPTION OF THE VARIETY CHINESE LONG

Cucumber plants of the variety Chinese Long are similar to plants of some of the White Spine varieties. The leaves average slightly larger, have a deeper green and have a more leathery or coarse texture than leaves of the Arlington Improved White Spine. The flowers also are usually larger, opening out with broad petals. The main point of differentiation is in the size and shape of fruits which in the Chinese Long are long and slender-necked, often curved or crooked like a crookneck squash. In the greenhouse

and field these fruits may grow to be 24 inches long and two to two and one-half inches thick at the greatest diameter. The fruits are not a pickling type, but they are satisfactory for slicing purposes. The fruits, when young, are well covered with white spines which shed as the fruits mature. The vines grow more rapidly in the greenhouse than White Spine plants, but in the field they do not branch so profusely. In China this variety is used almost exclusively as a climbing cucumber, where it produces an abundance of green fruits, which ripen yellow.

Bailey (2) states that the fruits of the variety *anglicus* produce few seeds, a condition confirmed by the writer's observations, but such a tendency has not been observed in the variety Chinese Long. The latter hybridizes readily with plants of both the White Spine and Chicago Special varieties, but two attempts to cross it with Rollison's Telegraph, representing the variety *anglicus*, failed.

It is evident that the Chinese Long cucumber is closely related morphologically to White Spine, Chicago Special and Davis Perfect and should be grouped in the species *Cucumis sativus* L. It is not a black spined variety, therefore it cannot be grouped with Long Green in Bailey's classification nor under the Giant Peru type because the fruits are green, ripening yellow. Physiologically, it is unquestionably different from all other varieties thus far studied in its reaction to mosaic.

#### PRODUCTION OF HYBRID CUCUMBERS

The undesirable fruit type of the Chinese Long for pickling purposes led to hybridization experiments with American sorts of the pickling fruit type. Dr. O. H. Elmer made the first crosses, using plants of the Arlington Improved White Spine variety as one of the parents.

The plants used for hybridization experiments were grown either in eight-inch pots or benched in soil. Practically all of the pollinating was done in the early morning between 5:30 and 7:30 soon after the flowers opened, using a small camel's hair brush to transfer the pollen from the staminate to the pistillate flowers. After pollination each fruit was tagged and the flower petals tied shut with a string. In most cases the flowers were tied shut before they were open to prevent insect visitation, but insects were not common and it was noticeable that fruits not artificially pollinated usually died from lack of fertilization.

#### THE F<sub>1</sub>, F<sub>2</sub> AND F<sub>3</sub> GENERATIONS

The first crosses between Chinese Long and White Spine were made with plants from open pollinated fruits, because no selfed lines were available. Because of the monoecious character of *Cucumis sativus* hybridization can be accomplished readily. The first hybrids were designated X1, X2, when the female parent was of the Chinese Long variety. The reciprocal cross was designated by R1, R2, . . . As soon as the fruits resulting from the cross were ripe the seed was removed, dried and a few seeds from several crosses planted in pots in the greenhouse. Three plants per pot of each hybrid were allowed to remain, of which two were inoculated with Cucumber virus 1 and the third one left as a check. The F<sub>1</sub> plants thus tested were X1, X3, X6, X8, X9, X11, X12 and R11. In each case all plants inoculated developed mosaic. The non-inoculated plants from each set were benched,



and one or two fruits were self-pollinated on each plant. Hybrid vigor in the  $F_1$  generation was pronounced, as evidenced by the rapid growth, large leaves and large fruits produced on the plants. The fruits produced were not all of the same shape or color. None of them were typical of the Chinese Long, nor were they short and cylindrical like those of the White Spine. They were more of an intermediate type between the two parents. The fruits ranged from seven inches in length and seven and one-half inches in circumference to 14 inches long by nine and one-half inches in circumference, at the greatest diameter. Most of the fruits were green, but a few on certain plants were either yellow or greenish white, a condition probably explained by discovering later that the Chinese Long parent in that cross produced both yellow and white fruits when selfed one generation. As a result of these tests, it appeared that susceptibility to mosaic was a dominant character, but that the fruit type of the  $F_1$  was intermediate. Later tests with  $F_1$  plants indicated that susceptibility might not be completely dominant. On April 4, 1928, 20 plants of X3 were inoculated and ten became infected. The infection on the checks was 76.2 per cent. Again, on May 1, 15 plants each of X21 and R9 were inoculated. In each case only two plants became infected.

Limited inoculation tests with plants of the  $F_2$ ,  $F_3$  and  $F_4$  generations resulting from the crosses referred to were carried out, but the numbers used were necessarily small and the trials were all conducted late in the fall at a time when cucumber plants do not grow well. Enough inoculations were made, however, to indicate that the factor or factors responsible for the mosaic resistant character in the Chinese Long variety could be segregated and combined to some extent with the pickling fruit type of the White Spine variety. More complete evidence of this indication was secured in tests involving backcrosses.

#### REACTION TO MOSAIC IN PLANTS FROM BACKCROSSES

In the spring of 1927 several backcrosses were made with  $F_1$  plants on each parent, using several combinations. The system used in designating the crosses is presented below in tabular form:

♀ ————— ♂		Cross designated by
X12	X12B	X12B
CL1	X7	CL1B1
CL11	R11	CL11B1
CL11	R11	CL11B2
R9	WS7	R9B'1
R9	WS7	R9B'2
R11	CL2	R11B1
R13	WS7	R13B'

The system used in designating the backcrosses is such that the original female parent is indicated in the first letter, for example, X12B is a backcross on the variety Chinese Long as male parent with an  $F_1$  in which the Chinese Long is the female parent. Also, R13B' is a backcross on the variety White Spine used as the male parent with an  $F_1$  in which White Spine is the female parent.

Beginning in May, 1928, seed from six of the backcrosses were planted in flats and the plants produced were inoculated in June with Cucumber virus 1. Several days after infection developed on part of the plants, the non-infected ones were reinoculated, transplanted to greenhouse benches and allowed to grow for several weeks in order to allow ample time for later development of mosaic. Plate I, figure 2, shows the response of plants from seed of R13B' just nine days after inoculation. Susceptible plants of this lot were badly stunted, but the non-infected plants made rapid growth. The final readings on all lots were made July 10 and the results are presented in table 8 in two parts, showing the response of backcrosses with  $F_1$  on varieties (1) Chinese Long and (2) White Spine.

TABLE 8. *Response to mosaic by cucumber plants from backcrosses, 1928*

Backcross number	No. plants inoculated	Date	Date final readings	No. infected	Ratio I:F <sup>3</sup>
CL11B1	29	6-12-28	7-10-28	13	0.81:1
CL1B1	30	"	"	16	1.14:1
X8B	29	"	"	13	0.81:1
X6B	30	6-13-28	"	17	1.3:1
R11B1	30	"	"	12	0.67:1
Total	148			71	0.92:1
R13B' <sup>2</sup>	30	6-12-28	"	21	2.3:1

<sup>1</sup> $F_1$  x CL.

<sup>2</sup> $F_1$  x WS.

<sup>3</sup>I = infected; F = free.

At the time the inoculations were made all plants in the greenhouse were in excellent growing condition and they continued so until late in July. It is evident that at least some of the inoculated plants which were non-infected were in reality resistant. On the basis of the results secured in the first part of table 8 it is indicated that mosaic resistance may be explained on the basis of a single factor difference segregating as a monohybrid ratio. This point needs further investigation with a larger population before any reliable conclusions may be drawn. It is of interest that when the backcross of  $F_1$  x White Spine was used, a higher percentage of infection took place than with  $F_1$  x Chinese Long. If mosaic resistance is explainable as a single factor difference and susceptibility completely dominant, all progeny of this backcross should be susceptible.

Further study of the reaction to mosaic in backcrosses was made during 1928, 1929 and in the spring of 1930. In table 9 the results of all the tests not given in table 8 are presented. In the summer of 1928 seed from one selfed fruit of a mosaic free plant of R13B' was saved and planted in the fall of 1929. All plants which resisted infection after the first inoculation were subjected a second time to an inoculation with mosaic virus. The total number of nine which remained free contained no virus, as shown by three unsuccessful attempts to cross-inoculate from each of these nine to plants of susceptible varieties on which no mosaic developed. No seed was saved from any of these nine plants because they did not mature. In the

spring of 1930 two sets of plants of this same strain, R13B'-1, and from the same seed lot were subjected to inoculation two and three successive times, respectively, and 14 plants out of 34 remained free from infection as far as outward symptoms were concerned. Plate V shows a plant of CL8-2 and two plants of R13B'-1 each with a fruit of the pickling type. One plant of the latter strain remained free from mosaic, the other became mildly infected.

An important condition noted in one test was the lack of stunting exhibited by infected plants. This was apparent in plants from the backcrosses of CL11B2 and CL1B1 shown in table 9. None of the 42 infected plants of CL11B2 and only one of the 30 infected plants of CL1B1 were stunted. This condition was similar to what occurred with both open pollinated and selfed lines of the variety Chinese Long parent and may be of significance since it was noted only when the  $F_1$  was backcrossed to the Chinese Long parent. This result may indicate that complete resistance to mosaic is established by complementary factors and that tolerant plants may possess most of the factors for resistance, but not enough to prevent the expression of mild symptoms of mosaic. McRostie (30), working with the inheritance to bean mosaic, found what appeared to be relative resistance. He advanced a two-factor hypothesis to account for the inheritance of resistance and susceptibility.

TABLE 9. *Reaction to mosaic by cucumber plants from backcrosses, 1928-1930*

Back-cross number	No. plants inoculated	Date	Date final readings	No. times inoculated	No. infected	Ratio I:F
CL11B2	49	7-27-28	8-12-28	1	42	6:1
CL1B1	47	7-27-28	8-12-28	1	30	1.77:1
R11B1	47	8-27-28	9-12-28	1	23	0.96:1
CL1B1	15	4-10-30	5-22-30	2	6	1.5:1
R11B1	20	4-10-30	5-22-30	1	10	1:1
Totals	178	( $F_1 \times CL$ )		1	111	1.66:1
R9B'1	23	4-10-30	5-22-30	1	12	1.09:1
R13B'-1	29	9-12-29	11-15-29	2	20 <sup>1</sup>	2.22:1
R13B'-1	14	3-30-30	5-22-30	3	6	0.75:1
R13B'-1	20	4-10-30	5-22-30	2	14	2.33:1
Totals	63	( $F_1 \times WS$ )			40	1.74:1

<sup>1</sup>Cross inoculations at three successive intervals were made from the nine resistant plants to *WS* plants. No infection took place at any time.

Some additional tests were made in the spring of 1931 using seed from the strain R13B'-1-9-1, a third generation selfed line from the backcross R13B'. A total of 26 plants were inoculated three different times at intervals of a week to 10 days, with the result that one plant became markedly stunted by mosaic, eleven were tolerant as indicated by faint mottling of the terminal leaves, vigorous growth and production of normal fruits, while the balance of 14 plants never showed any signs of mosaic. All of the plants save the stunted one produced fruits similar in type to those of the White Spine variety.

The data secured do not completely explain the behavior of the mosaic resistant character, but the evidence indicates that resistant plants can be segregated from a backcross and that by continued inbreeding the short fruit type may be combined with a high degree of mosaic resistance.

#### SUMMARY

The cucumber variety, Chinese Long, was found to be highly resistant to the virus of "white pickle mosaic" (Cucumber virus 1). A small percentage of the population became infected to a moderate degree, but such plants were decidedly tolerant to the virus, exhibiting no retardation in growth. Several inbred lines showed 30 to 55 per cent infection with no loss of vegetative vigor, following inoculation.

Tolerant plants often outgrew the symptoms and in such instances the virus was recovered by cross inoculation to known susceptible plants. At two different times mosaic was successfully transferred from inoculated plants on which no symptoms were observed. Faint to prominent mottling and in one case curling of the terminal leaves were the most common symptoms of mosaic on tolerant plants. Such symptoms as "white pickle" and stunting were not observed.

A new type of mosaic virus, designated as Cucumber virus 2, was secured from the Davis Greenhouses near Davenport, Iowa. Seven varieties of watermelon, the African citron, Green Seeded citron, West India gherkin (*Cucumis anguria*), and the Chinese Long cucumber were found to be highly susceptible to this virus. Stunting and mottling were common symptoms in the greenhouse, but in the field the plants often outgrew the symptoms.

The incubation period of Cucumber virus 2 is from two to four days longer than Cucumber virus 1 in White Spine plants. Stunting was not so pronounced on White Spine plants with this new type of mosaic as with "white pickle mosaic" and in no case were symptoms detected on the fruits when Cucumber virus 2 was used.

Cucumber virus 2 may be the same virus briefly described by Jagger (26) in 1917, but no data bearing on this point were secured. The present indication is that the geographical distribution of this virus is limited.

Plants of the variety Chinese Long are morphologically similar to plants of the variety Arlington Improved White Spine except in fruit shape, which in the former variety is long and slender. Many fruits were two feet in length. It is evident that this variety should be classed as *Cucumis sativus* L.

Hybridization between the varieties Chinese Long and White Spine was readily accomplished. Susceptibility to mosaic was found to be dominant in most of the inoculation trials and the fruit type of the  $F_1$  was intermediate, resembling more closely that of the White Spine parent.

Segregation for resistance and susceptibility apparently occurred in the  $F_2$ ,  $F_3$  and  $F_4$  generations and in the first generation from the backcrosses. When the backcross of  $F_1 \times$  Chinese Long was used the segregates were more commonly resistant and tolerant plants.

Nine plants out of 29 of the selection R13B'-1 resisted infection with the "white pickle virus" following two inoculations, and three attempts to recover the virus from these nine plants failed. The fruits on these plants



were of the short pickling type, indicating that the combination of the characters for mosaic resistance and pickling fruit type had occurred.

No reliable ratios bearing on the behavior of the mosaic resistant character were obtained.

## LITERATURE CITED

1. BAILEY, L. H.  
1919. Standard encyclopedia of horticulture, 2:907.
2. ————  
1925. Manual of cultivated plants. The MacMillan Co., New York, 851 pp.
3. BENNETT, C. W.  
1927. Virus diseases of raspberries. Mich. Agr. Exp. Sta. Tech. Bull. 80.
4. BRANDES, E. W.  
1919. The mosaic disease of sugar cane and other grasses. U. S. Dept. Agr. Bull. 829:1-26.
5. ————  
1924. Mosaic's role in limiting sugar yields in Louisiana. Facts About Sugar, 18:610-611.
6. BRIERLY, W. B.  
1915-1916. A case of recovery from mosaic disease of tomato. Ann. Appl. Biol., 2:236-266.
7. CARSENER, E.  
1925. Attenuation of the virus of sugar beet curly-top. Phytopath., 15:745-756.
8. ————  
1926. Resistance in sugar beets to curly-top. U. S. Dept. Agr. Cir. 388.
9. DOOLITTLE, S. P.  
1916. A new infectious mosaic disease of cucumbers. Phytopath., 6:145.
10. ————, AND W. W. GILBERT  
1919. Seed transmission of cucurbit mosaic by the wild cucumber. Phytopath., 9:326-327.
11. DOOLITTLE, S. P.  
1920. The mosaic disease of cucurbits. U. S. Dept. Agr. Bull. 879:1-69.
12. ————  
1921. The relation of wild host plants to the over-wintering of cucurbit mosaic. Phytopath., 11:47.
13. ————  
1923. Cross inoculation studies with cucurbit mosaic. Science, 57:477.
14. ————, AND M. N. WALKER  
1925. Further studies on the over-wintering and dissemination of cucurbit mosaic. Jour. Agr. Res., 31:1-57.
15. EDGERTON, C. W.  
1920. A method of selecting L511 cane free of the mosaic disease for planting purposes. La. Agr. Exp. Sta. Bull. 176:1-7.
16. ————  
1925. Selfing for resistance to the sugar cane mosaic. Phytopath., 15:45-46 (Abst.).
17. ELMER, O. H.  
1922. Mosaic cross-inoculation and insect transmission studies. Science, 56:370-372.

18. \_\_\_\_\_.  
1924. Mosaic cross-inoculation studies. *Phytopath.*, 14:55.
19. \_\_\_\_\_.  
1925. Transmissibility and pathological effects of the mosaic disease. *Iowa Agr. Exp. Sta. Bull.* 82:39-91.
20. \_\_\_\_\_.  
1927. A mosaic resistant variety of cucumbers. *Phytopath.*, 17:48 (Abst.).
21. FERNOW, K. H.  
1925. Interspecific transmission of mosaic diseases of plants. N. Y. (Cornell) *Agr. Exp. Sta. Memoir* 96.
22. FOLSOM, DONALD  
1920. Potato mosaic. *Maine Agr. Exp. Sta. Bull.* 292:157-184.
23. HOGGAN, ISME A.  
1929. The peach aphid (*Myzus persicae* Sulg.) as an agent in virus transmission. *Phytopath.*, 19:109-123.
24. \_\_\_\_\_.  
1930. Aphid transmission of plant viruses. *Phytopath.*, 20:133 (Abst.).
25. JAGGER, I. C.  
1916. Experiments with cucumber mosaic disease. *Phytopath.*, 76:148-151.
26. \_\_\_\_\_.  
1917. Two transmissible mosaic diseases of cucumber. *Phytopath.*, 7:61.
27. \_\_\_\_\_.  
1918. Hosts of white pickle mosaic diseases of cucumber. *Phytopath.*, 8:32 (Abst.).
28. \_\_\_\_\_.  
1918. Mosaic disease of cucurbits. *Phytopath.*, 8:74-75.
29. JOHNSON, JAMES  
1927. The classification of plant viruses. *Wis. Agr. Exp. Sta. Res. Bull.* 76.
30. McROSTIE, G. P.  
1921. Inheritance of disease resistance in the common bean. *Jour. Am. Soc. Agron.*, 13:15-32.
31. MELHUS, I. E.  
1922. Mosaic studies. *Phytopath.*, 12:42 (Abst.).
32. MOGENDORF, N.  
1930. "Fern-leaf" of tomato. *Phytopath.*, 20:25-46.
33. MURPHY, P. A.  
1921. Some recent work on leaf roll and mosaic. *Royal Hort. Soc., (London)* 145-152.
34. NISHAMURA, M.  
1918. A carrier of the mosaic disease. *Bull. Torrey Bot. Club*, 40:219-233.
35. PORTER, R. H.  
1928. Further evidence of resistance to cucumber mosaic in the Chinese cucumber. *Phytopath.*, 18:143 (Abst.).
36. \_\_\_\_\_.  
1929. Reaction of Chinese cucumbers to mosaic. *Phytopath.*, 19:85 (Abst.).
37. \_\_\_\_\_.  
1930. The resistance of cucumbers to mosaic. *Phytopath.*, 20:114 (Abst.).
38. \_\_\_\_\_.  
1930. A new mosaic disease of cucumber. *Phytopath.*, 20:113 (Abst.).

39. Rands, F. D., and S. F. Sheerwood  
1927. Yield tests of disease resistant sugar canes in Louisiana. U. S. D. A. Cir. 418:1-19.
40. Reddick, Donald, and V. B. Stewart  
1918. Varieties of beans susceptible to mosaic. Phytopath. 8:530-534.
41. Schultz, E. S., and Donald Folsom  
1923. Transmission, variation and control of certain degenerative diseases of Irish potatoes. Jour. Agr. Res., 25:43-117.
42. Smith, Loren B.  
1920. Breeding mosaic resistant spinach and notes on malnutrition. Va. Truck Exp. Sta. Bull. 31 and 32:137-160.
43. Verwoerd, Len  
1929. Mosaic disease of tomato plants. Ann. App. Biol., 16:1-2.
44. Walker, M. N.  
1926. A comparative study of the mosaic diseases of cucumber, tomato and Physalis. Phytopath., 16:431-458.

## PLATE I

Fig. 1. Symptoms of Cucumber virus 2 and Ringspot virus.

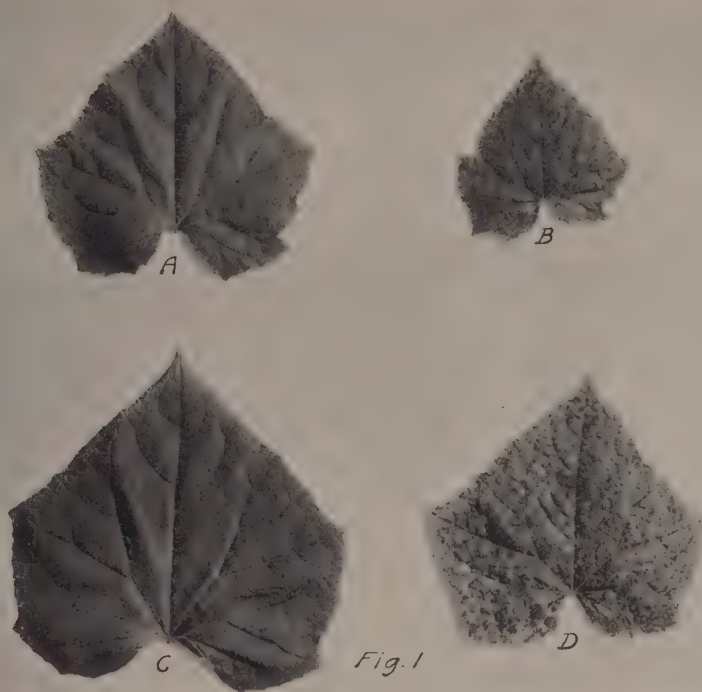
a and c, healthy.

b, Cucumber virus 2.

d, Ringspot virus.

Fig. 2 Segregation of susceptible and resistant plants of the backcross R 13 B'. In this test 4 plants out of 15 remained free from mosaic after inoculation. They are tall and apparently healthy. Infected plants are short and stunted.





## PLATE II

Fig. 1. Reaction of cucumber plants to Cucumber virus 1.

- a. Chinese Long
- b.  $F_1$  hybrid (Chinese Long x White Spine)
- c. White Spine

Fig. 2. Reaction of cucumber plants to Cucumber virus 2.

- a. Chinese Long.
- b.  $F_1$  hybrid (Chinese Long x White Spine)
- c. White Spine.



## PLATE III

Fig. 1. Effect of Cucumber virus 2 on watermelon plants.

- a. Infected (left) showing stunted growth and non-infected (right) plants of the variety Phinney's Improved.
- b. Infected (left) showing stunted growth and non-infected (right) plants of the variety Mountain Sweet.

Fig. 2. Effect of Cucumber virus 2 on citron plants.

- a. A non-infected plant of the Green Seeded citron.
- b. An infected plant of the Green Seeded citron.
- c. Infected (right) showing stunted growth and non-infected (left) plants of the African citron.





## PLATE IV

Fig. 1. Effect of Cucumber virus 2 on Tom Watson watermelon.

a. Leaf from healthy plant.

b and c. Leaves from an infected plant.

Fig. 2. Effect of Cucumber virus on leaves of citron and watermelon plants.

a, b and c. Left to right, leaves from infected plants of African citron, Green Seeded citron and Phinney's Improved watermelon, respectively.

d, e and f. Duplicate of a, b and c, except all leaves are from non-infected plants.



## PLATE V

Types of fruits on cucumber plants resistant and susceptible to Cucumber virus 1.

- a. Chinese Long
- b. Resistant plant of backcross R 13 B'1.
- c. Moderately susceptible plant of backcross R 13 B'1.







# EIMERIA SEPARATA, A NEW SPECIES OF COCCIDIUM FROM THE NORWAY RAT (*EPEMYS NORVEGICUS*)<sup>1</sup>

ELERY R. BECKER AND PHOEBE R. HALL

From the Department of Zoology and Entomology, Iowa State College

Accepted for publication September 27, 1931

The most recent discussion of rat coccidiosis appears to be that by Pérard (1926)<sup>2</sup>. According to this author, the correct name of the rat coccidium which has been observed by a number of workers is *Eimeria miyarii* Ohira, 1913. The oocysts of this protozoon are, in general, slightly egg-shaped, and measure on an average 18  $\mu$  in breadth and 23  $\mu$  in length. The sporulation process, requiring from two to four days, results in the formation of four sporocysts each containing two sporozoites and a residual body, but no residual body remains outside the sporocysts.

In August of this year we obtained near Ames a half grown Norway rat which, upon examination of its intestinal contents, was found to be infected with coccidia. By means of subsequent reinfections it was learned that this wild rat was actually infected with two markedly distinct species of *Eimeria*. From this material we have succeeded in obtaining pure infections with *Eimeria miyarii* in three white rats, pure infections with another *Eimeria* in three others, and mixed infections in seventeen others.

The predominant shape of the new coccidium is ellipsoidal, often approaching the spherical, but it was found that practically all the apparently spherical forms revealed themselves as slightly elongated when they were rolled about by tapping lightly on the coverglass. Ovoidal forms also exist. Measurements on 150 oocysts from one infected rat indicate a size range of from 13.1 to 23.8  $\mu$  in length and from 11.4 to 18  $\mu$  in breadth. The average size is 18 by 14.6  $\mu$ . The sporulation process, which is completed in less than 36 hours under optimum conditions, results in the production within each oocyst of four spores, each containing two sporozoites and a residual body, but no oocyst residual body is left.

Oocysts appear in the fecal pellets of the rat on the fifth day after the feeding of the sporulated forms. This interval, called by Andrews the prepatent period, is seven days in the case of *Eimeria miyarii*. The duration of elimination of oocysts by the animal, called by Andrews the patent period, is not over four days when the infection is produced by a single feeding and reinfection is prevented. The greatest numbers of oocysts are produced on the second day, while on the fourth day exceedingly few can be found. The patent period in the case of *E. miyarii* is five or six days, when the infection is produced by a single infective dose. The smaller species also appears to be a poor multiplier, for we do not encounter the oocysts in the enormous numbers found in *E. miyarii* infections.

For the new species we propose the name *Eimeria separata*. A more complete discussion of the coccidia of the rat will be published later.

<sup>1</sup>The new species described in this note was discovered during our researches on coccidiosis supported by a grant from the Rockefeller Fluid Research Fund at Iowa State College.

<sup>2</sup>Pérard, Ch. 1926. Sur la coccidiose du rat. Rec. Méd. Vét. Bul. et Mém. Soc. Méd. Vét., 102:120-124.





## HYDROGENATION OF FURFURAL

F. E. BROWN, HENRY GILMAN AND RALPH L. VAN PEURSEM

*From the Department of Chemistry, Iowa State College*

Accepted for publication November 5, 1931

### INTRODUCTION

The catalytic hydrogenation of furfural presents a wide variety of interesting possibilities and problems. In reducing this compound, the hydrogen may enter either the side chain or the furan nucleus. The aldehyde group may be reduced to the alcohol or to methyl furan, or it may be entirely removed, yielding furan. By hydrogenation of the furan ring, the products mentioned above may be converted into tetrahydrofurfuryl alcohol, dihydro- and tetrahydro-methyl furan and dihydro- and tetrahydro-furan, respectively. Rupture of the ring at the oxygen atom is also possible. This reaction may yield methyl propyl ketone, n-amyl alcohol, pentanediol-1, 2, and pentanediol-1, 5.

### SEPARATION OF PRODUCTS

Since so many different products may be formed in this reduction, the separation of these compounds presents a problem of its own. Various methods were tried and the reliability of each determined.

Societe Anon. des Destilleries des Deux-Sevres state in their patent<sup>1</sup> that furfural and water form a constant boiling mixture, which possesses a minimum boiling point of 97°, and that furfuryl alcohol does not show this property. This method was employed for some time in separating the reduction products. Methyl furan, if present, was removed by ordinary distillation. The mixture was steam distilled, as above, until the volume reached about 50 cc. The remaining furfural was removed by sodium bisulfite, and the furfuryl alcohol separated by ether extraction. This extract was fractionally distilled at a pressure of 50-70 mm.

The results obtained, when this method of separation was used, failed to show a consistent agreement. Consequently, the reliability of the separation was tested. Mixtures of furfural and furfuryl alcohol in varying proportions were steam distilled as described in the patent. In each case, the first few fractions distilling showed the presence of furfuryl alcohol, when tested with  $\alpha$ -naphthyl isocyanate.

The separation of furfuryl alcohol by fractional distillation was also attempted and tested for accuracy. This method gave a fairly quantitative separation, but was not considered reliable. It was found, however, that if the volume was reduced to about 50 cc. by vacuum distillation before the sodium bisulfite and ether treatment, the results gave very good agreement and almost 100 percent recovery was possible.

### IDENTIFICATION OF FURFURYL ALCOHOL

The identification of the products of the reduction of furfural amounts practically to the identification of furfuryl alcohol, since it is the principal

<sup>1</sup>Societe Anon. des Destilleries des Deux-Sevres, Fr. 639, 756, Jan. 31, 1927.

reduction product obtained. Furfuryl alcohol was identified by the preparation of a number of its esters. Since it is unstable in the presence of acids and acid compounds, its esters cannot be prepared by ordinary methods.

The furfuryl ester of diphenylcarbamic acid was prepared by heating one gram of furfuryl alcohol, 2 g. of diphenylcarbonyl chloride and 1.3 g. of pyridine on a water bath for one hour under a reflux condenser. The resulting compound, when crystallized from alcohol, melted at 98°. The yield was approximately 75 per cent of the theoretical. This compound has been described<sup>2</sup>.

The furfuryl ester of  $\alpha$ -naphthyl carbamic acid was made by heating one gram of furfuryl alcohol and 1.8 grams of naphthyl isocyanate for a few minutes. On cooling, the urethane crystallized out. When crystallized from petroleum ether, this compound melted at 130°. The yield was about 90 per cent. This compound has been prepared by Bickel and French<sup>3</sup>.

Mono-furfuryl phthalate was prepared according to the following method. Sixty grams of toluene (dried over sodium) and 20 grams (0.2 mole) of furfuryl alcohol were placed in a 250 cc. flask fitted with a reflux condenser. To this mixture was added slowly 4.5 grams (0.2 gram-atom) of sodium, which had been cut up into small pieces. After all of the sodium had been added, the mixture was refluxed for one hour. The sodium furfurylate, which settled, was filtered out by means of suction. The sodium furfurylate and 27 grams (0.18 mole) of phthalic anhydride and 375 grams of toluene were placed in a liter three-necked flask fitted with a mechanical stirrer and reflux condenser. The mixture was stirred for one hour, heated to boiling, and refluxed for 45 minutes. It was filtered by suction and air-dried. It was stirred with 500 cc. of water for several minutes and filtered. The filtrate was acidified with hydrochloric acid (1-5) and the mono-furfuryl phthalate was filtered by suction. Since this compound had not been previously described in the literature, a complete analysis was made.

*Anal.* Subs., 0.2591: Calcd. for  $C_{13}H_{10}O_5$ : C, 63.41; H<sub>2</sub>, 4.07. Found: C, 63.43; H<sub>2</sub>, 4.18.

The saponification equivalent of the compound was obtained.

*Anal.* Subs., 0.2118: Calcd. for  $C_{13}H_{10}O_5$ : Sapon. Equiv. 246. Found: Sapon. Equiv., 245.

The compound was saponified. The acid, which was recovered, was identified as phthalic acid by: (1) the fluorescein test and (2) preparation of the amide, which melted at 220°. Monofurfuryl phthalate, when pure, melted at 85° and decomposed at 135°.

#### PREPARATION OF CATALYST

The copper catalyst was prepared from the sulfate. A quantity of c.p. copper sulfate large enough to contain ten grams of copper was dissolved in about four liters of water. While this was continuously stirred with a mechanical stirrer, ninety grams of acid-washed asbestos was slowly added. After the asbestos was uniformly distributed throughout the solution, the solution was made alkaline with approximately three normal

<sup>2</sup>Erdmann, Ber., **35**, 1855-1862 (1902).

<sup>3</sup>Bickel and French, J. Am. Chem. Soc., **48**, 747-751 (1926).

sodium hydroxide. The mixture was filtered and allowed to become air dry. The nickel catalyst was prepared in a similar manner. The proper amount of nickel carbonate was dissolved in concentrated nitric acid. This solution was diluted to about four liters and the nickel precipitated on asbestos as the hydroxide by means of sodium hydroxide.

## METHOD OF PROCEDURE

In each run, the catalyst tube was stocked with 25 grams of the prepared catalyst, which was equivalent to about 2.5 grams of nickel or copper. The catalyst was heated to 200° until water vapor no longer was given off and then hydrogen was passed through the tube until no more water vapor was formed. The catalyst was then brought to the desired temperature and the reduction started. The hydrogen-furfural mixture was secured by bubbling hydrogen through a supply of furfural heated to a temperature near its boiling point. The temperatures are indicated by the first column of the tables labelled "Bath." The mixture was not permitted to cool before it was brought into contact with the catalyst. The reduction was continued until 300 cc. of distillate had been collected.

Runs were made with nickel at 200°, copper at 175°, 200°, 225°, 250° and 275°. A series of eight runs with nickel as catalyst was made and analyzed by the steam distillation method. No reduction products were found in any mixture. The temperature, hydrogen, furfural and catalyst were all changed, but no effect was noticeable.

The method of analysis was tested as shown. In all subsequent runs, the last method of analysis was used. Runs were made with copper at 200°, 215°, 225°, 235° and 250°. The results of the runs are shown in tables 1 and 2.

## EXPERIMENTAL DATA

TABLE 1. *Catalyst—nickel*

Temperature° C.		H <sub>2</sub> cu. ft.	Dist. cc.	Furfuryl alcohol	
Bath	Cat.			cc.	pctg.
151	200	4.2	300	15	5

TABLE 2. *Catalyst—copper*

Temperature° C.		H <sub>2</sub> cu. ft.	Dist. cc.	Time min.	Furfuryl alcohol	
Bath	Cat.				cc.	pctg.
153	175	5.67	300	300	trace	
152	200	4.4	300	395	6.0	2.0
151	200	4.5	300	250	6.5	2.17
150	215	4.8	300	240	13.0	4.33
152	225	6.36	300	300	12.0	4.0
152	225	6.31	300	350	11.0	3.67
150	235	5.2	300	260	8.0	2.67
153	250	6.7	300	255	trace	
150	250	5.3	300	265	4.0	1.33
154	275	4.86	300	260	0	0

## SUMMARY

Furfural can be reduced by passing its vapors, mixed with hydrogen, over nickel or copper heated to temperatures of 175°-275°. The yield is not large enough to warrant utilization of this method for large scale production of the reduction products at the present state of development.

The chief product of the reduction of furfural is furfuryl alcohol. It is possible that the reaction proceeds farther, although no other products were isolated.

The amount of furfuryl alcohol produced is dependent upon the temperature. Under the conditions of this work, the temperature at which the production of furfuryl alcohol reaches a maximum is 215° or very near it. It is possible that there are other factors which affect the amount of reduction and optimum temperature.

One run with nickel gave a larger yield of furfuryl alcohol than copper, since the yield with nickel at 200° was larger than was obtained from copper at the optimum temperature. Nickel, on the other hand, is much more easily poisoned and consequently cannot be used except under conditions in which no impurities are introduced.



# THE PHYSIOLOGICAL PROPERTIES OF SOME FURAN DERIVATIVES

HENRY GILMAN, A. P. HEWLETT AND J. B. DICKEY

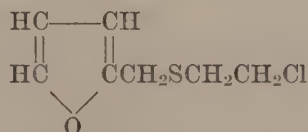
*From the Chemical Laboratory of Iowa State College*

Accepted for publication November 23, 1931

## INTRODUCTION

In connection with a series of studies concerned with the utilization of furfural and derivatives, we have incidentally noticed that several compounds have repellent physiological properties. These properties make it possible to classify the compounds roughly as sternutators (sneeze-provoking compounds), lachrymators (tear-inducing compounds) and vesicants (skin-blistering compounds).

When compounds with such objectionable properties have been encountered they have been carefully examined pharmacologically in order that exact information might be available for ascertaining the precautions which should be exercised in handling them. Toxicity tests have also been made. The results of some of these tests are now reported. The vesicant action of  $\beta$ -chloroethyl furfuryl sulfide



was reported earlier<sup>1</sup>, and two interesting observations were made in connection with that study. First, the homologous  $\gamma$ -chloropropyl furfuryl sulfide is without vesicant action. Second, the  $\beta$ -chloroethyl furfuryl sulfide, in undiluted form, provoked intense red spots but no blisters on hogs, whereas, solutions of relatively low concentrations induced blisters on human skin. The experiments on hogs, carried out with the assistance of Professor M. D. Helser, were made in connection with the possible utility of these and other compounds for the commercial marking of hogs.

It should be emphasized that there is nothing inherently offensive physiologically to furan types. Derivatives of furfural, like the furylacrylic esters<sup>2</sup>, can have unusually pleasant and attractive bouquets which commend them for perfumes and flavoring extracts; and some are highly unattractive, like the evil-smelling mercaptans<sup>3</sup>. It is a curious fact that some of the offensive mercaptans induce a pleasant physiological response in low concentrations and have been recommended as synthetic coffee flavoring extracts because of the pronounced coffee-like odor which they have when highly diluted and properly blended.

<sup>1</sup>Gilman and Hewlett, *J. Am. Chem. Soc.*, **52**, 2141 (1930).

<sup>2</sup>Gilman and Wright, *Iowa State College Jour. Sci.*, **3**, 109 (1929).

<sup>3</sup>Gilman and Hewlett, *ibid.*, **5**, 19 (1930).

Furthermore, many furan types have promising pharmacological properties which indicate their application as local anaesthetics<sup>4</sup>, blood sugar lowering agents<sup>5</sup>, analgesics, hypnotics, etc.<sup>6</sup> Some furan compounds, like sodium furylacrylate, are used as preservatives for chop suey sauce condiments; other furan compounds are unusually sweet, one (*syn.*-5-benzyl-2-furfuraldoxime) being sweeter than saccharin<sup>7</sup>. The physiological action of these sweet-tasting compounds has not been determined.

Finally, it should be emphasized that a great number of furan compounds which are apparently innocuous to man are relatively toxic to insects, and the results of these studies on flies will be published shortly in this Journal.

#### EXPERIMENTAL PART

*Tetrahydrofurfuryl Chloroacetate*,  $C_4H_7OCH_2OCOCH_2Cl$ .—In a first preparation, 40.8 g. (0.4 mole) of tetrahydrofurfuryl alcohol and 37.8 g. (0.4 mole) of chloroacetic acid were added to 100 cc. of benzene saturated with dry hydrogen chloride. The solution was treated with hydrogen chloride from time to time over a period of fifty hours. After the benzene had been removed by distillation, the residue was taken up in ether and washed with 5 per cent sodium bicarbonate solution, and dried over sodium sulfate. The yield of tetrahydrofurfuryl chloroacetate, distilling at 101–104°/4 mm., was 35 g. or 49.3 per cent. The ester is a stable water white liquid of pungent taste and practically no odor. It is soluble in ether, alcohol and benzene, but insoluble in water.

*Anal.* Calcd. for  $C_7H_{11}O_3Cl$ : Cl, 19.86. Found: Cl, 20.01 and 20.00.

In a second preparation, 33.9 g. (0.3 mole) of chloroacetyl chloride was added dropwise to a boiling solution of 30.3 g. (0.3 mole) of tetrahydrofurfuryl alcohol in 250 cc. of benzene, contained in a three-necked flask equipped with mechanical stirrer, reflux condenser and dropping funnel. After refluxing until evolution of hydrogen chloride ceased, the benzene was removed under diminished pressure, and the ester distilled. The yield of ester, distilling at 110°/5 mm., was 52.5 g. or 99 per cent.

*Tetrahydrofurfuryl Iodoacetate*  $C_4H_7OCH_2OCOCH_2I$ .—Thirty g. (0.2 mole) of sodium iodide was added to a solution of 35.7 g. (0.2 mole) of tetrahydrofurfuryl chloroacetate in 200 cc. of alcohol. The mixture was warmed, with vigorous stirring, for two hours and the alcohol removed under diminished pressure. The addition of 50 cc. of anhydrous ether caused the separation of sodium salts which were removed by filtration. The filtrate was distilled to yield 35 g. or a 65 per cent yield of tetrahydrofurfuryl iodoacetate boiling at 130°/5 mm. It is a colorless liquid which darkens on standing.

*Anal.* Calcd. for  $C_7H_{11}O_3I$ : I, 47.21. Found: I, 46.99 and 47.14.

---

<sup>4</sup>Gilman and Pickens, *J. Am. Chem. Soc.*, **47**, 245 (1925).

<sup>5</sup>Unpublished studies by Mr. H. J. Harwood and Mr. F. Prochaska.

<sup>6</sup>Details on these studies will be published later.

<sup>7</sup>Gilman and Dickey, *J. Am. Chem. Soc.*, **52**, 2010 (1930). See, also, Gilman and Hewlett, *Iowa State College Jour. Sci.*, **4**, 27 (1929).

*Furoyl Chloride*,  $C_4H_3OCl$ .—The following directions which are adapted from those by Gilman and Hewlett<sup>8</sup> not only give a high yield of furoyl chloride, but also permit operation with a minimum of discomfort to the experimenter. Whenever a choice is to be had in the use of either furoic acid or furoyl chloride for a given reaction, it should be remembered that the 75 per cent yield of furoyl chloride is higher than the yield of pure furoic acid obtainable by crystallization of the technical furoic acid at present available.

In a two-liter distilling flask is placed a mixture of 224 g. (2 moles) of crude furoic acid, 357 g. (3 moles) of technical thionyl chloride and one liter of benzene. After corking the side-arm, the flask (equipped with a reflux condenser) is placed at an angle of  $45^\circ$ , in a sand bath. The preparation is preferably carried out in a hood. The mixture is refluxed moderately for 14-48 hours, at a sand bath temperature of about  $140^\circ$ . The flask is then attached to a water condenser and the benzene removed by distillation, after which a two-holed stopper with thermometer and capillary-bubbling tube is inserted in the neck of the flask. When the temperature of the distilling vapor reaches  $95^\circ$  the pressure is reduced to 35 mm.; the residue is distilled with an oil bath or free flame, finally increasing the heating temperature (if an oil bath is used) to  $200-250^\circ$ . A first fraction of benzene should be collected. The yield is 195 g. or 75 per cent distilling at  $84^\circ/35$  mm. If the furoyl chloride is not to be used immediately it should be redistilled.

*5-Chlorofuroyl Chloride*,  $ClC_4H_2OCl$ .—A 65.25 g. (0.5 mole) portion of furoyl chloride was placed in a flask and one gram of iron powder added. The mixture was heated at  $100^\circ$  and a stream of dry chlorine passed through. The mixture gradually became yellow in color and finally began to darken, becoming deep red in color. At this point, without determination of increase in weight, the reaction was stopped and the mixture fractionated at 10 mm. pressure. The fraction boiling at  $92-110^\circ/10$  mm. was collected and refractionated to yield 55 g. or 66 per cent of material boiling at  $92-95^\circ/10$  mm. No constant boiling fraction could be separated from the higher boiling material, which formed no amide. A small amount of the fraction boiling at  $92-95^\circ$  was dissolved in ether, and ammonia gas passed through. The amide thus secured, when crystallized from alcohol, melted at  $154^\circ$ , which corresponds to the melting point given by Hill and Jackson<sup>9</sup> for 5-chlorofuramide ( $ClC_4H_2OCONH_2$ ). A small amount of the 5-chlorofuroyl chloride was dissolved by refluxing with a slight excess of 10 per cent sodium hydroxide. The resulting solution was just neutralized with hydrochloric acid and concentrated by evaporation. The resulting solution was then cooled and made acid with hydrochloric acid to yield an acid melting at  $177^\circ$ . A mixture of this acid and that prepared by the method of Hill and Jackson<sup>9</sup> melted at  $177^\circ$ .

---

<sup>8</sup>Gilman and Hewlett, *Iowa State College Jour. Sci.*, 4, 29 (1930).

<sup>9</sup>Hill and Jackson, *Am. Chem. J.*, 12, 22 (1890).

## PHYSIOLOGICAL PROPERTIES

*Tetrahydrofurfuryl Chloroacetate.*

Pure compound on skin of man: no reaction.

As a lachrymator: 0.0065 mg./l., irritation, but no lachrymation.

Lethal point for mice, 10 min. exposure:  $>2.5$  mg./l.

*Tetrahydrofurfuryl Iodoacetate.*

Pure compound on skin of man: redness.

As a lachrymator: 0.0035 mg./l., slight irritation.

Lethal point for mice, 10 min. exposure:  $>2.5$  mg./l.

*Furoyl Chloride.*

Pure compound on skin of man: no reaction.

As a lachrymator: 0.0035 mg./l., slight irritation.

Lethal point for mice, 10 min. exposure:  $>2.5$  mg./l.

*5-Chlorofuroyl Chloride.*

Pure compound on skin of man: no reaction.

As a lachrymator: 0.0065 mg./l., slight lachrymation.

Lethal point for mice, 10 min. exposure:  $>2.5$  mg./l.

*5-Nitrofurfural Chloride*<sup>10</sup>,  $\text{NO}_2\text{C}_4\text{H}_2\text{OCH}_2\text{Cl}$ .

The lethal concentration for mice on ten minutes exposure was found to be greater than 7 mg./l. Comparative vesicant tests indicated that mustard gas,  $(\text{ClCH}_2\text{CH}_2)_2\text{S}$ , was approximately 150 times as powerful as the 5-nitrofurfuryl chloride.

In connection with the standard value of mustard gas as a vesicant, it is also desirable to add that chloroacetophenone ( $\text{C}_6\text{H}_5\text{COCH}_2\text{Cl}$ ) in a concentration of 0.0004 mg./l. lachrymates in 194 seconds. Accordingly, it is evident that the compounds tested are very much less powerful than the standard lachrymators.

These quantitative relative values are interesting because of the light they throw on subjective approximations. For example, a number of students who have worked with both chloroacetophenone and furoyl chloride were very strongly of the opinion that the furoyl chloride was more powerful as a lachrymator. Even now, despite the accuracy of the results just reported, some of them persist in their contention. It is not at all unlikely that their opinions might be influenced by secondary or auxiliary physiological reactions, as well as by the high persistency of furoyl chloride.

The present report would be incomplete without some mention of purely qualitative observations which it is hoped may be of value to other workers with furan compounds. Furoic acid, particularly as a powder, is irritating to mucous membrane and has distinct sternutating properties. Furfuryl chloride,  $\text{C}_4\text{H}_3\text{OCH}_2\text{Cl}$ , has lachrymatory properties and appears to be generally irritant to the eyes, throat and nose. 2, 5-Dinitrofurran,  $\text{NO}_2\text{C}_4\text{H}_2\text{ONO}_2$ , has a vesicant action<sup>11</sup>. Other nitro compounds, so far investigated, appear to be without vesicant action. However, constant contact

<sup>10</sup>The 5-nitrofurfuryl chloride was prepared by the direct nitration of furfuryl chloride and from 5-nitrofurfuryl alcohol. Its chemical reactions and details of preparation will be reported later.

<sup>11</sup>The properties of this compound, as well as its improved preparation by the nitration of furoyl chloride and furoic acid will be described later.



with them appears to form a complex with the skin protein, which is horny and brittle. The skin then cracks, leaving a condition remindful of chapping.

Chloromethyl furfural,  $\text{ClCH}_2\text{C}_4\text{H}_2\text{OCHO}$ , is a skin irritant which with some produces redness and sores, particularly at the more tender places between the fingers. It is possible that a part of this action is due to the spattering of concentrated hydrochloric acid, large volumes of which are handled in such syntheses.

An unusual case is a compound which might be a dibromofurfural, and which analyzes for  $\text{C}_5\text{H}_2\text{O}_2\text{Br}_2$ . This compound has no immediate effect when placed on the hands. If an alcohol solution of the dibromo compound is allowed to dry on the hands, even though the hands are then thoroughly washed with soap and water, large blisters form after twenty-four hours. These are decidedly uncomfortable, although they heal rapidly after being broken. An aqueous solution of sodium carbonate will remove the compound from the hands where soap and water fail.

It should be remembered in all these physiological properties that the susceptibilities of individuals vary markedly, as is the case with many non-furanic types.

A general treatment for such skin irritants might be added, finally. It is well to remember, however, that antidotes for poisons are a good deal out of style. There are really extremely few safe and effective antidotes mentioned in works on pharmacology. The chief emphasis should be placed upon prevention; everything should be regarded as potentially dangerous, and great care must be exercised not to get compounds on the skin or clothes, or to inhale the vapors. One reason why the use of antidotes is so often hopeless is that many of the poisons (phenol, for example) soon form difficultly reversible compounds with the tissues. A general treatment, particularly for acidic materials, is as follows:

"First neutralize the causative material, or remove it with solvent. Wash the part thoroughly with water, and an alkalinized water is best, such as bicarbonate solution. This should be done very thoroughly, and the part should be immersed in the alkaline solution for a period of from 10 to 15 minutes. This treatment is often preventive and keeps blisters and the resultant ulcers from forming. Should blisters form, they should be evacuated by the use of a sterile needle, pricking the blisters in two places, but being careful to keep the epidermis intact. Of course, care should be taken that these are not infected, and a sterile dressing may be advisable for protection. Should the blisters be large and break down, a raw, ulcerated surface occurs. This is liable to infection. Dakin's solution used twice daily as a wash is advisable, and is perhaps better than many of the other antiseptics. Healing of the ulcers may be stimulated by the employment of scarlet red powder; of course an antiseptic dressing should be used."

The authors gratefully acknowledge assistance from G. F. Wright, R. R. Burtner, N. O. Calloway, W. H. Zugschwerdt and E. V. Brown.

#### SUMMARY

A description is given of the lachrymatory, vesicant and sternutatory properties of some furan compounds encountered incidental to studies on furfural and derivatives.



# CERTAIN CHEMICAL AND MORPHOLOGIC PHASES OF THE BLOOD OF NORMAL AND CHOLERA-INFECTED SWINE<sup>1</sup>

## I. THE CONCENTRATION OF CERTAIN CHEMICAL CONSTITUENTS

E. A. HEWITT<sup>2</sup>

*From the Department of Veterinary Physiology and Pharmacology, Iowa State College*

Accepted for publication December 15, 1931

The raising of swine is a major industry in the United States. Many of the problems confronting this industry lend themselves to physiologic study.

The metabolism of the pig occupies a unique position in mammalian physiology inasmuch as this animal excels in the economy with which it converts food into body tissue. It is evident that the growth impetus is greater in the pig than any other domestic mammal. Henry and Morrison (84) state that for every four or five pounds of dry matter ingested as food, the pig will increase one pound in body weight. Pigs at birth weigh on an average of two and one-half pounds. When fattened for the market they should have at least one pound of weight for every day of age. For pigs weighing under 50 pounds the average gain per day is 0.8 pound, and this gradually increases until at 250 to 300 pounds weight they may show a daily gain of 1.5 pounds.

The pig is excelled by no other domestic mammal in fat storing ability and yields from 70 to 80 per cent of its live weight as a dressed carcass.

For these reasons the pig presents an animal of unusual physiologic interest. A review of the literature discloses but few studies on the physiology of swine.

One of the outstanding hazards to profitable swine husbandry is the disease of hog cholera. This disease is of great economic importance. It is widely spread and is extremely contagious. It is usually fatal to susceptible swine. Hog cholera has been the subject of extensive investigations by various workers, and many valuable contributions have added to our knowledge of this disease. Studies of the blood of normal swine or of cholera-infected swine have been but little pursued. Even in the case of man, few extensive analyses have been performed on the same sample of blood. It is felt that as many analyses as possible performed on the same blood sample would be desirable, as it would give better data for determining correlations between different constituents.

This lack of knowledge as to the composition of the blood of swine has been keenly felt by veterinary physiologists. These studies were undertaken with the idea that such data as were obtained would be a significant

---

<sup>1</sup>These data are taken from a thesis presented to the Graduate Faculty, University of Minnesota, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>2</sup>The writer is indebted to Dr. H. D. Bergman for providing facilities and offering helpful suggestions for this research.

contribution to our present knowledge of the physiology of the blood of normal swine and thus help to interpret some of the pathologic phenomena occurring in the disease of hog cholera.

The results reported in this paper are divided into two parts. Part I deals with the concentration of certain chemical constituents in the blood of normal and cholera-infected swine. Part II considers certain morphologic phases of the blood of normal and cholera-infected swine.

Accurate data on the chemical composition of the blood, especially the non-protein fraction, are of comparatively recent origin. This phase of study has been developed within the past two decades. Among the men outstanding for this development are Otto Folin, S. R. Benedict and D. D. Van Slyke.

The development of the technique for blood analysis has been a great aid to the physiologist and the clinician. A great deal of information is available dealing with the abnormal composition of human blood in disease. Obviously, a knowledge of the composition of normal blood has been a necessary prerequisite to the interpretations of these abnormalities.

Studies of the chemistry of the blood give information concerning deranged physiology. In human medicine there is no laboratory procedure that will give more dependable information and aid in diagnosis, prognosis and treatment of disease than the chemical analysis of the blood. Whether this will prove equally true in veterinary medicine, only exhaustive researches will determine.

Additional data are necessary, not only for the purpose of establishing the normal physiologic composition of the blood of domestic animals, but also for the purpose of using the examination of the blood of diseased animals as an aid to diagnosis, prognosis and treatment. We recognize many conditions in animals which are analogous with conditions in the human to which valuable applications of blood analyses have been made.

#### REVIEW OF LITERATURE

An extensive literature dealing with the blood of farm animals is not available. A review of the literature shows that little work has been done on the chemical composition of either the normal swine blood or on the blood of cholera-infected swine.

Considerable literature has been reviewed relative to the composition of human blood and that of various domestic animals.

In 1912 Folin and Denis (55), described colorimetric methods for the determination of total non-protein nitrogen and urea of the blood. They used acetone-free methyl alcohol as the protein precipitant. In the same year Folin and MacCollum (59) called attention to the possibilities of the use of phosphotungstic acid in the colorimetric estimation of uric acid. A few months later in 1912 Folin and Denis (54) gave the first description of a colorimetric method for the estimation of uric acid in blood. The coagulation of the blood proteins was effected by the use of weak (0.01N) acetic acid. The following year analogous methods were described by Folin (51) for the determination of creatinine and creatine.

Imperfect as the earlier forms of these micro-methods of blood analysis were, their application revealed at once fundamentally important facts, which earlier investigators using improvised methods had sought but could not find.



## HUMAN BLOOD

The system of blood analysis, as described by Folin and Wu (61, 62), has placed in the hands of investigators a means of studying the changes taking place in the concentrations of the various blood constituents in health and disease. The literature of the chemistry of the blood is scattered through a number of periodicals, and contains many reports of the normal limits within which these substances fluctuate in the human.

Hammett (77) studied the non-protein nitrogen and non-protein nitrogenous constituents in human blood. The average of sixty determinations on normal individuals was as follows: Total non-protein nitrogen 35.6 milligrams per 100 c. c.; urea nitrogen 17.1 milligrams per 100 c. c. or 47.8 per cent of the total non-protein nitrogen; creatinine nitrogen 0.47 milligrams per 100 c.c. or 1.3 per cent of the total non-protein nitrogen; uric acid nitrogen 0.78 milligrams per 100 c.c. or 2.2 per cent of the total non-protein nitrogen. In addition, Hammett studied the composition of the blood in relation to the time of withdrawal. He found no practical difference in the concentration of the various constituents determined in blood which had been taken three and one-half and fourteen hours after eating. From a metabolic point of view the blood taken before breakfast showed evidence of a slightly lower metabolism at this period. The non-protein nitrogen, the urea nitrogen, and the uric acid nitrogen were consistently lower, but to a very small extent. He found that the sugar might be somewhat higher after a longer fast.

Hammett further studied the range of variations, average amounts, average deviations and relative variability of the blood constituents, with the following results: Creatinine nitrogen ranged between 0.37 and 0.60 milligrams, with an average of 0.47 milligrams per 100 c.c. of blood. The average deviation was 7.3 per cent and the relative variability 100 per cent. The non-protein nitrogen ranged between 27.3 and 45.5 milligrams, with an average of 35.6 milligrams per 100 c.c. of blood. The average deviation was 9.1 per cent and the relative variability was 125 per cent. The sugar ranged from 85.0 to 166.0 milligrams, with an average of 112.0 milligrams per 100 c.c. of blood. The average deviation was 13.7 per cent and the relative variability 188 per cent. Uric acid nitrogen ranged between 0.50 and 1.16 milligrams, with an average of 0.78 milligram per 100 c.c. of blood. The average deviation was 14.9 per cent and the relative variability 204 per cent. Urea nitrogen ranged between 9.7 and 25.1 milligrams, with an average of 16.6 milligrams per 100 c. c. of blood. The average deviation was 16.6 per cent, with a relative variability of 227 per cent.

This writer stated that the significance of the position of the various constituents in this table was not wholly obvious. They could be, however, roughly divided into three groups, one of relatively low variability containing the creatinine and the total non-protein nitrogen, one of intermediate variability, into which would fall the sugar, and one of relatively high variability which would contain the uric acid and urea nitrogen.

Folin and Berglund (53) determined the normal minimum, maximum and average blood content of non-protein nitrogenous products from 12 young men after a night's fast. The values for urea nitrogen were between a minimum of 8.9 milligrams and a maximum of 15.2 milligrams, with an average of 11.5 milligrams per 100 c.c. of blood. The values for total non-protein nitrogen were between a minimum of 27.8 milligrams and a maxi-

imum of 39.4 milligrams, with an average of 32.1 milligrams per 100 c.c. of blood. The amino acid nitrogen values were between a minimum of 5.7 milligrams and a maximum of 7.8 milligrams, with an average of 6.4 milligrams per 100 c.c. of blood. The undetermined rest nitrogen values were between a minimum of 10.1 milligrams and a maximum of 17.5 milligrams, with an average of 13.7 milligrams per 100 c.c. of blood. The undetermined nitrogen included uric acid nitrogen, creatinine and creatine nitrogen with a trace of ammonia.

A comprehensive survey of the chemical and physical composition of human blood is that of Gettler and Baker (65). They made chemical and physical analyses of the blood of thirty normal individuals. The values they obtained for non-protein nitrogen ranged between 30 and 45 milligrams per 100 c.c. of blood. Three cases went a trifle higher. They stated that all values above 50 should be regarded as pathologic. The values for urea nitrogen ranged between 15 and 25 milligrams per 100 c.c. of blood. Two cases fell below 1 milligram per 100 c.c., showing 0.7 and 0.6 milligrams, respectively. Most of the values obtained for preformed creatinine were 0.1 milligram or less for 100 c.c. of blood. For creatine the values ranged between 3.0 and 6.5 milligrams. For sugar the values obtained ranged between 50 and 120 milligrams per 100 c.c. of blood.

Gettler and Baker (65) compared the results of their chemical analyses with the results obtained by various workers. The results are given in milligrams per 100 c.c. of blood. Non-protein nitrogen: Gettler and Baker 30-45; Folin and Denis 22-37; Greenwald 30; Bang 19-39; Taylor and Hulton 25-28; McLean and Selling 23-44; Myers and Fine 25-30; Holweg 40-61. Urea nitrogen: Gettler and Baker 15.0-25.0; Folin and Denis 12.0-27.0; Bang 6.0-20.0; McLean and Selling 12.0-27.0; Myers and Fine 12.0-15.0; Schwartze and McGill 10.8-25.2. Uric acid: Gettler and Baker 1.0-3.5; Folin and Denis 0.7-3.7; Myers and Fine 1.0-2.0; Maase and Zondek 2.5. Creatine: Gettler and Baker 3.0-6.5; Folin and Denis 5.2-8.1; Myers and Fine 5.0-10.2. Creatinine: Gettler and Baker 0.1-0.5; Folin and Denis 1.1-1.4; Myers and Fine 1.0-2.01. Sugar: Gettler and Baker 50-120; Folin and Denis 90-110; Bang 100-110; Taylor and Hulton 50-150; Michaelis 90-130; Strouse 40-120; Naunyn 70-100; Liefman and Stern 70-110; Hollinger 70-110; Frank 80-110; Rolly and Opperman 62-88; Kowarsky 50-110; Freund and Marchand 55-120.

Myers (118) states the following values for different blood constituents per 100 c.c. of normal human blood. Non-protein nitrogen 25 to 35 milligrams; urea nitrogen 12 to 15 milligrams; uric acid 2 to 3 milligrams and creatinine 1 to 2 milligrams. The sugar values ranged from 0.09 to 0.12 per cent. The states "that it is difficult to draw an arbitrary line indicating where normal findings end, and pathologic begin, but it is believed safe, when blood is taken after a fourteen hour fast (in the morning before breakfast), to regard urea nitrogen above 20 milligrams per 100 c. c. and a sugar value above 0.15 per cent as quite definitely pathologic."

#### BLOOD FILTRATES

Folin and Wu (61) in 1919 described a method for the preparation of a protein free blood filtrate in which tungstic acid precipitated the proteins. The protein-free blood filtrates so prepared lend themselves perfectly to the non-protein nitrogen determinations by the direct Nesslerization process of Folin and Denis (56).

### *Non-protein Nitrogen*

Folin and Wu (61) introduced certain modifications of the Folin-Denis (56) method for the determination of total non-protein nitrogen which they believed represented improvements.

Koch and McMeekin (102) described a modification of the Folin-Denis method for the determination of non-protein nitrogen in which one to five drops of a thirty per cent hydrogen peroxide solution is added to the sulphuric acid digestion mixture, after it has been heated over a micro-burner until the dense white fumes have filled the tube and then allowed to cool for fifteen to thirty seconds. This method avoids the troublesome separation of silicon dioxide so common in the Folin-Denis (56) procedure and shortens the time of digestion.

Folin (50) stated that the unknown nitrogenous products in blood filtrates are greatly increased in those cases in which there is excessive retention of nitrogen. In such bloods the undetermined nitrogen is more abundant in the plasma than in the corpuscles. It is not possible to accurately define the nitrogenous materials contained in blood filtrates, which make up the total non-protein nitrogen. These products contained in the filtrates can be classified into three groups: (1) the nitrogenous waste products, (2) the absorbed nitrogenous food materials, and (3) undetermined materials, including some undetermined waste products, some undetermined absorbed food products, and in addition some products of unknown origin.

### *Urea*

Probably the first definite proof that urea exists in the blood was furnished by Prevost and Dumas (128) in 1821. They described certain experiments in which both kidneys were removed from animals such as dogs, cats and rabbits. Analysis of the blood of such animals showed the presence of a large amount of urea, which was identified by determining its properties and by performing an elementary analysis.

Marshall (110) in 1913 described a method for the determination of urea in blood which consisted of a conversion of urea into ammonium carbonate by means of the enzyme urease of the soybean, and a subsequent removal of the ammonia by means of an air current. By this method satisfactory results could be obtained without a preliminary removal of the proteins, as was necessary in other methods for the determination of urea in blood.

Folin and Wu (61) described a method for the determination of urea nitrogen in the tungstic acid blood filtrates by means of urease solution. Folin (49) used a urease preparation in the form of filter paper impregnated with a strong urease solution. The ammonia from the urea may be obtained by distillation or by an air current. He also described a method of decomposing the urea of the blood filtrate by heating under pressure.

Numerous modifications of this method of urea determination have been suggested, among them, Youngberg (156) used an open delivery tube in distillations when determining urea and non-protein nitrogen. This overcame the difficulty of sucking back of the distillate into the heated flask. Johnson (99) used a flutter valve to prevent the same thing. Gruskin (70) added the urease to the whole blood before filtering the proteins. Butka and Meisner (32) described technical improvements in the ammonia distillation



by the use of an ebullition tube in the distilling fluid, a small capillary pipette which controlled the rate of admission of air, a water jacket, and a capillary outlet for gasses into the acid solution. The aspiration was accomplished by means of a small filter pump attached to a water outlet. Cooper (39) prevented foaming in the urea solution, during the removal of ammonia by aeration, by the use of a mixture of 1 c. c. of toluol and 0.02 c. c. of caprylic alcohol. By this procedure the Nesslerized solutions remained clear for one hour. Roe and Irish (133) described a method for urea determination in which the urease was added to the whole blood and then a protein free filtrate was made by tungstic acid. Sevringhaus and Hipple (140a) stated that the ammonia contained in the filter paper and the ammonia in the water constituted two sources of error in blood urea and nitrogen determinations.

Karr (100) described a method for blood urea nitrogen determination in the tungstic acid filtrate by urease conversion, Nesslerization and colorimetric comparison without removal of ammonia from the digestion mixture. An artificial standard solution was used composed of a mixture of iron, cobalt and nickel chlorides, previously standardized against a known urea solution. The artificial standard occasionally became cloudy, perhaps caused by deposition of ferric salts. They found that the artificial standard should be freshly prepared. When only a few determinations were to be made and urease paper used, it was possible and perhaps preferable to read the unknown against the color developed from the standard urea solution instead of the artificial standard.

Marshall and Davis (111) stated that urea was present in all the organs and tissues. The amount present in the tissues was approximately uniform and about equal to that of the blood, both in normal conditions and when there was an abnormally large amount of urea present. The tissues which formed an exception to this rule were fat, which had a low urea content, and those of the urinary tract, which had a high content of urea.

When urea in solution was injected intravenously, it diffused to all parts of the body almost instantly.

The urea was eliminated rapidly by the kidneys. The rate of excretion rose in some cases to 16 grams per kilo of body weight per day or even much higher. The rate of excretion of urea in normal animals was directly proportional to the concentration of urea in the blood. It was sometimes retarded, however, by a dehydration of the organism.

Mosenthal and Hiller (115) stated that the body usually metabolized protein in such a manner that approximately 80 per cent of the nitrogen set free in the blood was in the form of urea. The selective action of the kidney maintained the urea nitrogen at a level of 50 per cent or less of the total non-protein nitrogen of the blood. An impairment of renal function even to a slight degree, sometimes resulted in an increase of the percentage of urea nitrogen. This increased percentage of urea nitrogen sometimes occurred whether the total non-protein nitrogen was high or low. Cases with acute renal conditions showed a high percentage of total non-protein nitrogen, as urea nitrogen of the blood, which returned to normal as convalescence ensued.

Individual patients, whose clinical condition did not vary appreciably, exhibited a constant percentage of urea nitrogen whether the total non-protein nitrogen was high or low.



Mosenthal and Hiller concluded that from a clinical point of view, figures for urea nitrogen were preferable to total non-protein nitrogen. The methods for urea nitrogen determination of the blood were simpler and were perfected so that they yielded constant results comparable to those of other observers. The methods of determining total non-protein nitrogen as used by different investigators did not always yield constant results.

The percentage of non-protein nitrogen occurring as urea nitrogen, as well as other non-protein nitrogen fractions in the blood required much more detained study in order to be of extensive clinical significance.

### *Uric Acid*

Garrod (64), as early as 1848, identified uric acid in the blood of patients suffering from gout. Moreover, this writer showed that this substance was normally present in the blood only in traces, and was definitely increased in gout and in certain cases of nephritis.

No noteworthy advance in our knowledge of the blood uric acid was made from the time of Garrod until Folin and Denis (54) in 1913 reported their first results on the uric acid content of human blood. In a series of unselected cases they (58) found between 1 and 3 milligrams per 100 c.c., the average being close to 2 milligrams. They divided the figures into three groups, arranged according to the amount of uric acid found. Group 1 consisted of 5 cases with figures ranging from 0.7 to 0.9, with an average of 0.8 milligrams per 100 grams of blood. Group 2 consisted of 22 cases ranging from 1 to 2 milligrams, with an average of 1.47 milligrams per 100 grams of blood. Group 3 consisted of 11 cases ranging from 2.3 to 3.7 milligrams, with an average of 2.83 milligrams per 100 grams of blood. They stated that in certain kinds of human blood (gout and lead poisoning) there was an accumulation of uric acid.

Myers (118, p. 51) stated that the figures now regarded as normal for uric acid content of human blood were between 2 and 3 milligrams per 100 c.c. of blood. Figures as low as 1 milligram and as high as 3.8 milligrams were sometimes encountered in strictly normal individuals, the difference probably depending in part upon dietary factors. Kingsbury and Sedgwick (101) observed that the blood uric acid was high in infants during the first three or four days of life, in harmony with the high uric acid excretion during that period.

In 1915 Benedict (12) introduced certain modifications which did much to increase the accuracy and simplicity of the Folin-Denis method (54) of uric acid determination. Potassium cyanide was employed to decompose the silver precipitate instead of hydrogen sulphide. Another modification recommended by Benedict and Hitchcock (15) was the preparation of a standard uric acid solution in which the uric acid was held in solution by a mixture of disodium hydrogen phosphate and sodium dihydrogen phosphate, instead of the formaldehyde uric acid solution of Folin and Denis. Benedict and Hitchcock also recommended the combined use of silver lactate, magnesia mixture and ammonia into a single reagent and the use of colloidal iron to remove the last trace of protein after coagulation.

Folin and Wu (61) in 1919 described a method of estimating uric acid directly in the protein-free blood filtrate obtained after tungstic acid precipitation without evaporation of the filtrate. They precipitated the uric

acid from the filtrate with a special lactic acid silver lactate reagent, after which the uric acid was set free from the silver precipitate by a solution of sodium chloride containing hydrochloric acid.

Benedict (10) developed an extremely simple technique whereby the uric acid color reaction was developed directly on the Folin-Wu tungstic acid blood filtrate. He employed a different uric acid reagent (arsenic-phosphotungstic acid) and used cyanide as the sole alkali and heat in the development of the color. Folin (52) considered that the success of Benedict's method was not in the uric acid reagent but the use of the cyanide as the sole alkali. Folin adopted most of Benedict's suggestions (49, p. 247) except that he continued to employ the original Folin-Denis uric acid reagent.

Brown and Raiziss (27) compared the Folin-Wu and Benedict methods of determining uric acid, and concluded that adsorption of uric acid did not take place in the precipitation of blood proteins by the method of Folin and Wu. Equally good recoveries of added uric acid could be obtained by either the Folin-Wu or Benedict method. The Folin-Wu method gave correct results for blood uric acid, while the high results found with Benedict's method were caused by interfering substances. When the blood contained large amounts of uric acid, Benedict's reagent was selective and yielded results similar to those of Folin and Wu. They further concluded that Benedict's method, owing to its speed and requirement of small quantities of blood, could be used for routine clinical analysis, but was not to be recommended for research purposes.

### *Creatinine and Creatine*

Jaffé (95) was the first to direct attention to the fact that when a solution of creatinine was added to a solution of sodium or potassium picrate containing an excess of alkali a deep orange red color resulted. He referred to the reaction as being due to the reduction of picric acid.

Practically no quantitative information on the creatinine and creatine of the blood was available until Folin (51) in 1914 introduced the colorimetric method for their determination and about the same time Folin and Denis (57) reported observations on a series of normal and miscellaneous cases and nine cases of uremia and two cases of nephritis. Creatinine in normal and miscellaneous cases ranged from 0.9 to 1.8 milligrams per 100 c.c., whereas in the uremia cases 4 to 32 milligrams per 100 c.c. was found. A case of chronic nephritis showed 7.5 milligrams and a case of nephritis hemiplegia coma showed 3 milligrams per 100 c.c. of blood. Myers (116) stated that for normal individuals the creatinine of the blood amounted to 1 to 2 milligrams per 100 c.c., the findings for the strictly normal being nearer 1 than 2 milligrams. As soon as one passed to hospital patients, however, higher values were found. Although the great majority of cases without renal involvement showed creatinine figures on the whole blood below 2.5 milligrams per 100 c.c., occasionally figures as high as 3.5 milligrams were usually accompanied by urea retention. Many of the cases below 4 milligrams sometimes showed improvement, but with over 4 milligrams the reverse was usually true.

Folin and Wu (61) described adaptations of the methods of determining preformed creatinine and creatine plus creatinine to the tungstic

acid protein-free blood filtrates. Folin (50) stated that the normal creatinine content of human blood could be given as 1.2 to 1.5 milligrams per 100 c.c. of whole blood, according to results obtained by the method of Folin and Wu. The creatine content of such blood varied between 3.5 milligrams and 5 milligrams. He further stated that the creatinine content of blood was remarkably constant normally, which was to be expected, in view of the fact that the endogenous production of creatinine was by far the largest source of the urinary creatinine.

Chase and Myers (36) emphasized the point that large retentions of creatinine represented the most valuable single index in advanced cases of nephritis in which there were nitrogen retentions.

Shaffer (141) stated that creatinine was derived from some special process of normal metabolism taking place, largely if not wholly, in the muscles. The intensity of this process appeared to depend upon the muscular efficiency of the individual. Creatine was not a normal product of endogenous metabolism and was not present in the urine unless the individual had taken creatine in the food. Creatine was sometimes excreted in acute fevers and in other conditions in which there was a rapid loss of muscle protein. The appearance of creatine in the urine probably indicated that muscle protein was being absorbed.

Shaffer stated that the number of milligrams of creatinine excreted in twenty-four hours per kilo of body weight is called the creatinine coefficient, and shows a direct parallelism with the muscular development of strength or "muscular efficiency" of the individual.

Hunter and Campbell (94) stated that the creatinine content of normal human blood plasma ranged under different conditions from 0.7 to 1.3 milligrams per 100 c.c. of blood. The average for 60 specimens examined was 1 milligram. It was practically certain that the creatinine of normal blood was distributed through corpuscles and plasma in uniform concentration. Creatinine was usually lower in females than in males and lower in subjects deprived of exercise. The creatine of the blood was concentrated chiefly in the corpuscles, the average content in the corpuscles being between 6 and 9 milligrams per 100 c.c., while the plasma contained not more than 0.4 to 0.6 of a milligram per 100 c.c. The blood as a whole contained approximately 3 milligrams of creatine per 100 c.c.

Wang and Dentler (150) reported observations on twenty-four normal women, fifteen of whom were observed during both the menstrual and intermenstrual period. No regular variation was found during menstruation for either creatinine or creatine. In nine cases there were slight creatinine increases during menstruation and in five cases there were decreases. On the other hand, seven cases showed lower and eight showed higher creatine values during menstruation.

The creatinine in the different subjects ranged from 0.96 to 1.65 milligrams, with an average content of 1.3 milligrams per 100 c.c. of blood. This fell within the range observed by other investigators and failed to substantiate the suggestion that blood creatinine was lower in women.

The creatine concentration was 2.23 to 4.65 milligrams per 100 c.c., which was slightly lower than the range observed by others. No relation was observed between the age of the subject and increased creatinine.

Myers (117) stated that the physiological fact to be born in mind in regard to creatinine, was the absolute constancy of its elimination, different



for different individuals, but wholly independent of the volume of urine. In a disease in which the creatinine output was low, creatine was generally excreted. Creatine was not normally present in urine and was to be regarded as pathologic. It was usually found associated with loss of muscle protein.

Feinblatt (45) reported observations on creatininemia based upon 1500 blood chemical analyses. The creatinine blood values exceeded 2.5 milligrams per 100 c.c. in forty-three patients. The analysis of this group showed that there were other unmistakable evidences of renal deficiency. Forty cases were diagnosed as glomerulonephritis, while the other three were anemic. Fourteen patients who had attained figures of 10 milligrams or over, died within 17 days, the average period having been four days. Of fifteen patients who yielded figures from 5 to 10 milligrams, eleven patients died within 17 days, the average period having been six days. Three others died later. Of twenty-one patients with readings ranging from 2.5 to 5 milligrams, sixteen died. The urea sometimes rose with the creatinine, but not always. They stated also that the accumulation of uric acid bore an inconstant ratio and its readings were of little value in forming a prognosis.

Hubbard (93) stated that the urea and creatinine values tended to vary together except when the urea nitrogen concentrations were low. The urea at times accumulated earlier in the blood than did creatinine, but often the creatinine later increased fairly rapidly to a comparable level.

Poyales (127) stated that the index of creatinine in the blood gave a prognostic value, especially in retinal lesions of renal origin. The elevation of the amount of creatinine in the blood of nephritics gave an index to proximity of death.

Sedgwick (140) reported that creatinine was present in the liquor Amnii, which possibly meant that its excretion began before birth. Creatinine was always present in the urine during the first week and in a concentration which approximately equalled that of adult urine. Creatine was also excreted during infancy.

### *Blood Sugar*

According to Myers (118, p. 79), Dobson in 1775 first recognized that blood contained a sugar-like substance. Claud Bernard (18) first demonstrated the presence of sugar in normal blood. It remained for Lewis and Benedict (107) to introduce a colorimetric method for blood sugar estimation, in which the red color obtained by heating a dextrose solution with picric acid and sodium carbonate was employed as the basis. The colored derivative formed was probably picramic acid. The blood proteins were removed by precipitation with picric acid. One objection to this method was the necessity of boiling the solution to dryness to complete the reaction between the sugar and the picric acid.

Pearce (125) modified the method by using the higher temperature of the autoclave to develop the color. Myers and Bailey (119) further modified the original method by providing for less dilution of the blood, so that the final reaction took place in a more concentrated mixture of glucose and picric acid. Benedict (11) further modified the method, using the same dilution as the original method, but using a solution of sodium picrate and



picric acid and developing the color by immersing the solution in boiling water for 10 minutes.

Folin and Wu (61) in their system of blood analysis, developed a method for the estimation of blood sugar adapted to the tungstic acid blood filtrates. They used an alkaline copper solution which was reduced by the sugar. A stable color reaction was obtained by the application of a special sugar reagent (phosphotungstic-phosphomolybdic acid) to the cuprous oxide. In 1920 Folin and Wu (62) published a modification of this method. They modified the preparation of the sugar reagent and recommended the use of a special "sugar test tube" for the determination.

Numerous modifications of the Folin-Wu method and the other methods have been made. Benedict (8) described a colorimetric method for the determination of blood sugar based on a copper citrate reagent. Folin (48) in 1926 further modified the method by using a new alkaline copper tartrate solution. He also used a new acid molybdate reagent for the estimation of cuprous copper. The blood filtrate was neutralized. Benedict (9) in 1928 described a method for the determination of blood sugar in which he added alanine to the copper tartrate solution.

Another method of sugar estimation that should be mentioned is that of Hagedorn and Jensen (75). The principle of this method is the precipitation of the blood proteins by means of zinc hydrate and oxidation of the sugar in the filtrate by potassium ferrieyanide. The amount of residual ferrieyanide may then be estimated by taking advantage of its power to liberate iodine from iodides.

According to Myers (116) the blood sugar of the normal human subject appeared to fall somewhere between 0.09 and 0.12 per cent, the average being approximately 0.10 per cent. The results were dependent upon the method employed for determination, at times differing by as much as 0.02 per cent in normal blood. Slightly higher figures seemed to be obtained by the picric acid method of Benedict in its various modifications, than by most of the other methods. It seemed certain that the reducing power of the blood was to a great extent caused by glucose, although various methods appeared to be influenced by other reducing substances. The figures obtained by the various methods, however, differed so little relative to the variations that occurred in disease that the question of the method scarcely entered into a discussion of the blood sugar findings in disease.

The figure 0.10 per cent for normal individuals applied to observations made in the morning previous to the intake of any carbohydrate. After a meal rich in carbohydrate there was at times an appreciable rise in the sugar content of the blood to 0.12 or 0.14 per cent. Foster (63) showed that the sugar content in arterial blood was materially higher than in venous blood. The sugar of the blood was the topic of an extensive review by MacLeod (108) in 1921.

Feinblatt (46) studied hyperglycemia based upon two thousand blood sugar determinations. Eighty-one determinations yielded readings in excess of 150 milligrams per 100 c.c., thirty-four patients or 42 per cent of this group presented clinical evidence of diabetes mellitus. The remaining forty-seven patients were considered non-diabetic. Of the non-diabetics the diagnosis accounted for the hyperglycemia in only fourteen, or 30 per cent. It was believed that a single report of an abnormally high blood sugar value could not be construed as pathologic, inasmuch as transient hypergly-

cemia often resulted from emotional disturbances. The renal blood sugar level varied in different individuals and in the same individual under different circumstances. In the diabetic a high threshold often existed.

Greisheimer (69) found that the irritability of the reflex arcs of decerebrate dogs increased as the blood sugar decreased. This occurred whether the blood sugar decreased spontaneously or after insulin. She suggested the probability that a fundamental relationship existed between the blood sugar level and irritability of the nervous system.

John (97) concluded from an analysis of 10,368 synchronous blood sugar and urea determinations that there was an apparent lack of dependence of one on the other.

### *Phosphorus*

The study of the occurrence and distribution of phosphorus compounds in blood has been stimulated by the introduction of new analytical methods. Bloor (20) and Buell (29) divided the phosphoric acid compounds of human blood into three distinct types: (1) Inorganic phosphates; (2) lipoids that contain phosphorus; and (3) organic substances other than lipoids that contain phosphorus. Classes (1) and (3) were referred to collectively as "acid soluble phosphates." Class (3) was termed "organic phosphate" or "unknown phosphate." The acid soluble phosphates and the lipid-phosphoric acid compounds were apparently sharply defined, in general their sum equaling the total phosphates. The presence of other forms of phosphoric acid combinations in blood in significant amounts was doubtful.

Bloor (20) gave the average content of inorganic phosphorus in the plasma of both men and women as about 3 milligrams per 100 c.c. and of lipid phosphorus about 7.5 milligrams. Buell (29) concluded that inorganic phosphorus was absent from the corpuscles. Bloor (20) states that, "the amount of the unknown form of phosphorus combination in the plasma is very small, but in the corpuscles it constitutes 60 to 80 per cent of the total phosphorus."

The presence of lipid phosphorus in the blood has long been recognized, but data regarding the inorganic phosphorus are of more recent origin. Clinical interest is attached at present to the inorganic phosphorus.

Greenwald (66) in 1915 observed that normally the acid soluble (largely inorganic) phosphorus varied between 2 and 6 milligrams per 100 c.c. of serum, but that in severe nephritis it was at times considerably increased. These observations were confirmed by Marriott and Howland (109).

Bloor (21) in 1918 developed a nephelometric method for the determination of phosphoric acid in blood, which was adapted from a method first introduced in this country by Greenwald (66). It consisted of ashing the blood by the use of sulphuric and nitric acids with heat. A strychnine molybdate reagent was used to react with the phosphates to form phosphomolybdate, which was compared against a standard in a nephelometer.

In 1914 Taylor and Miller (144) described a method for the determination of phosphates which was adapted from the Neumann method (121) for titrimetric estimation of phosphoric acid. This method depended on the precipitation of ammonium phosphomolybdate and the subsequent colori-

metric determination of the molybdenum in the precipitate. This reaction was based upon the following equation:



Bell and Poisy (7) in 1920 found that by reducing molybdic acid with phenol (hydroquinone) in carbonate sulfite solution, a blue color was obtained which was proportional to the amount of phosphorus present. Because of the rapid fading of the alkaline blue solution, Briggs (24) suggested that the reading be made in acid solution. Briggs (25) also modified the method by increasing the strength of the hydroquinone solution from 0.5 to 1 per cent. Benedict and Theis (17) combined the hydroquinone and bisulfite into one solution. Fiske and Subbarow (47a) substituted 0.25 per cent solution of aminonaphthol-sulphonic acid for the hydroquinone solution.

Denis and Minot (43) in 1920 found, in conditions other than nephritis and cardio-renal disease, figures for inorganic phosphorus varying from 1.2 to 3.1 milligrams per 100 c.c. of plasma in the human. However, in one case of uremia figures exceeding 40 milligrams were observed.

DeWesselow (44) reported observations on fifty-three cases of nephritis with thirteen deaths. In twelve of the cases that died the highest inorganic phosphorus figures ranged from 10 to 22 milligrams per 100 c.c. of plasma. He believed that phosphorus retention was more definitely connected with the symptoms of true uremia than was the retention of urea. Schmitz, Rohdenburg and Myers as stated by Myers (116) collected observations on a comparatively large series of nephritics, the highest figure for inorganic phosphorus being 25.5 milligrams. In several cases low figures for calcium—4 to 5 milligrams—were encountered, the phosphorus values being about 15 milligrams. These cases showed uremic convulsions.

Howland (91) summed up the findings on infants and stated that the breast fed infant had an average of about 6 milligrams of inorganic phosphorus per 100 c.c. of serum. The average for the artificially fed child was a little lower, about 5.5 milligrams, while the rachitic infant had 3.5 milligrams or less.

Tisdall and Harris (147) and Myers with his collaborators (113) observed that following major fractures in adults there was a rise in the inorganic phosphorus content of the blood, in many instances to the level found in children. At times the rise took from several days to two or four weeks. After union of the fracture the phosphorus gradually fell. As a rule, cases with non-union following fracture did not show this reaction in the blood phosphorus.

Byrom and Kay (33) stated that in the normal individual the inorganic phosphorus was only one-twelfth of the total phosphorus. In disease the retention of inorganic phosphorus was roughly proportional to the severity of the disease. However, a marked retention of phosphorus appeared, to begin later than urea retention, which these writers considered a serious prognostic sign.

Tolstoi (148), in studying the phosphorus of serum and plasma of ninety-one normal adults, concluded that the inorganic phosphorus ranged from 2.5 to 3.3 milligrams per 100 c.c.



### Calcium

According to Clark (38) the methods for the determination of calcium, if classified according to the manner of obtaining solutions from which calcium may be precipitated, fell into three groups: (1) Destruction of the organic matter (proteins) by ashing or by digestion and solution of the ash in hydrochloric acid; (2) Precipitation of the proteins, by picric, tungstic or trichloroacetic acids, and the use of aliquot parts of the filtrate; (3) Direct precipitation, in the presence of proteins, so far only applied to whole blood, plasma and serum.

Although every method utilized the oxalate ion for the precipitation of calcium, considerable variation was observed as to the manner of obtaining the hydrogen ion concentration at which pure calcium oxalate was most completely precipitated. After obtaining the washed calcium oxalate the following procedures were used for the actual estimation of the calcium.

1. Gravimetrically, by conversion to calcium oxide or calcium sulphate and weighing.
2. Volumetrically (a) by titration with potassium permanganate, and (b) by solution in excess acid and titration of excess acid.
3. Nephelometrically, by conversion into a calcium soap.
4. Colorimetrically, by decolorization of ferric thiocyanate with oxalates.
5. Iodometrically by solution of calcium oxide in excess hydrochloric acid, the excess acid then determined by estimating the amount of iodine liberated.  $5\text{I}^- + 10\text{O}_3^{2-} \pm 6\text{H}^+ \rightarrow 3\text{H}_2\text{O} + 4\text{I}_2$ .

Clark described also a micro-method for the determination of calcium which depended on the direct precipitation of calcium from serum, plasma and whole blood, as calcium oxalate and titrating with 0.01 normal potassium permanganate. The procedure required several hours for a determination.

Kramer and Tisdall (105) in 1921 described a simple method for determining calcium in serum by precipitating the calcium as calcium oxalate in a centrifuge tube. The precipitate was driven to the bottom by the use of the centrifuge and was subsequently washed three times with two per cent ammonia. The tube was centrifuged for five minutes after each washing. After the last washing the supernatant fluid was syphoned off. The crystals were suspended in the residual liquid and dissolved in 2 c.c. of normal sulphuric acid, heated in a water bath and titrated against 0.01 normal potassium permanganate to a definite pink color, which persisted for at least a minute. In 1923 Tisdall (146) simplified the technique of washing the precipitate.

Clark and Collip (37) modified the procedure as given by Tisdall by substituting one washing with 3 c.c. of ammonia for two washings with 4 c.c. of dilute ammonia. The tubes were allowed to drain for five minutes, which reduced the amount of mother liquor left in the tube from about 0.1 c.c. to 0.02 c.c. Thus, the loss of calcium due to its solubility was balanced by the amount of ammonium oxalate left in the precipitate. This method was accurate within two per cent.



The literature on the variations, which occur in the concentration of calcium in blood under normal conditions, is not extensive.

Myers stated (118) that the calcium content of human blood serum was normally constant at somewhere between 9 and 11 milligrams per 100 c.c. of serum, the figures averaging slightly higher for children than adults. It has been recognized for some time that the tetany following parathyroidectomy was associated with a decreased blood calcium and that the symptoms of tetany were relieved by calcium therapy. Myers stated that in infantile tetany the calcium content fell to between 3.5 and 7 milligrams per 100 c.c.

Koechig (103) reported that in various pathologic conditions the calcium content of the plasma did not differ from the normal values, which were between 9.5 and 11 milligrams per 100 c.c. Low figures have been found in nephritis, colitis, pellagra, jaundice, osteomalacia and tetany.

Wells (155) reported studies on blood calcium and inorganic phosphates in children with marked lack of muscle tone. The children were divided into three groups. Group 1 consisted of ten normal children, who had a serum calcium value ranging from 10.1 to 11 milligrams, with an average of 10.4. Group 2 consisted of twenty cases of inflamed tonsils and adenoids, but a good muscle tone, the calcium values ranged from 8.7 to 10.6 milligrams, with an average of 9.8. Group 3 consisted of thirty cases showing a marked lack of muscle tone, and having calcium values ranging from 6.6 to 10.6 milligrams, with an average of 9. This author concluded that the blood picture in flabby children was not similar to that of rachitic children, although the calcium variation was the same in both conditions.

Hess and his associates (85) stated that in moderate rickets the phosphorus or calcium, or both, might be moderately increased. In severe rickets the phosphorus was markedly reduced even in those cases exclusively breast fed, while calcium might or might not be lowered. In rickets complicated by tetany the calcium was considerably reduced. The phosphorus might or might not be reduced.

Anderson (5) stated that such variations as occurred in rachitic calcium values had no significance, either as an indication of the severity or as an indication of activity or healing. She concluded that, although the blood calcium was slightly more variable in rachitic than in normal children, this variability had no prognostic nor diagnostic value, and a study of calcium values alone was unlikely to prove of much assistance as an indication of cause or treatment.

Peters and Eiseron (126) stated that the concentration of calcium in serum varied directly with the concentration of protein and inversely with the concentration of inorganic phosphorus.

Halverson, Mohler and Bergeim (76) found that there was no appreciable difference in the calcium content of plasma and serum. They found that the normal calcium content ranged between 9 and 11 milligrams per 100 c.c. of plasma, whereas in tuberculous patients it ranged from 8.4 to 11 milligrams. As a result of the experiences of the writers the indications were that the calcium content of the serum was little affected by the calcium given with the food.

Danielopolu and Maxim (41) stated that there was a slow increase of calcium in the blood in attacks of angina pectoris with 12.5 milligrams per 100 c.c. of plasma during the attack and 14.1 milligrams after the attack.

## THE CONCENTRATION OF CHEMICAL CONSTITUENTS IN THE BLOOD OF ANIMALS

*The Blood of Laboratory Animals*

A review of the literature on the composition of the blood of animals shows that no exhaustive study has been made in this field since 1898, when Abderhalden (1) published a rather complete analysis of the blood of various domestic animals.

In 1913 Folin and Denis (58) in the presentation of their early micro methods (54, 55), gave a table for the non-protein nitrogen, urea nitrogen and uric acid nitrogen of the blood of the horse, sheep, pig and the ox. The following year they published figures for creatinine and creatine in the blood of the ox, sheep and pig, using the picric acid method (51, 57). In 1915 Benedict (13), modifying the procedure of Folin and Denis, studied the uric acid present in the blood of the ox. Greenwald (67) and Greenwald and McGuire (68), testing the efficiency of trichloroacetic acid-kaolin as protein precipitates, published figures for the non-protein nitrogen and creatinine of the blood of the dog, sheep and ox.

*Dogs.* The chemical composition of the blood of dogs has received more attention than that of any mammal other than man.

Haden and Orr (71) reported the chemical findings in the blood of twenty-five normal dogs. The average non-protein nitrogen was 30.3 milligrams, urea nitrogen 11.1 milligrams, uric acid 1.5 milligrams, creatinine 1.5 milligrams, amino-acid nitrogen 6.3 milligrams, sugar 76 milligrams, and chlorides 458 milligrams per 100 c.c. of blood. They found that after intestinal obstruction there was a rise in urea nitrogen, non-protein nitrogen and sugar. They stated that there was a close similarity of the blood findings, in intestinal obstruction, acute lobar pneumonia and serum disease, which suggested that these widely different conditions may have had a common chemical basis. Similar changes were noted after pyloric obstruction (73) and after obstruction of the esophagus and cardiac and of the stomach (72).

Haden and Orr (74) also reported the results of two hundred consecutive analyses of the blood of normal dogs. The average non-protein nitrogen was 30.8 milligrams, urea nitrogen 11.7 milligrams, creatinine 1.5 milligrams, the amino-acid nitrogen 6.7 milligrams, and the sugar 82 milligrams per 100 c.c. of blood.

Morgulis and Edwards (114) reported the normal composition of dog blood from the examination of six dogs which ranged in weight from 9.58 to 19.86 kilos. The non-protein nitrogen varied between 31.9 and 40 milligrams per 100 c.c., with an average of 34.8 milligrams per 100 c.c. The urea nitrogen varied between 11.1 and 15.1 milligrams and averaged 12.9. The amino-acid nitrogen varied between 7.8 and 9.5 milligrams, with an average of 8.7. The creatinine values were between 1.5 and 1.7 milligrams per 100 c.c., with an average of 1.5 milligrams per 100 c.c. The creatine ranged between 2.4 and 4 milligrams, with an average of 3.16 milligrams per 100 c.c. of blood. The sugar varied from 74 milligrams to 106.3 milligrams, with an average of 96.7 milligrams per 100 c.c. of blood. The undetermined nitrogen varied from 26 to 44 milligrams, with an average of 31.5 milligrams per 100 c.c. of blood.

Morgulis and Edwards have compared the values of certain constituents in the blood of the dog, which have been obtained by different authors.

The results are given in milligrams per 100 c.c. of blood. Non-protein nitrogen: Bang 34.0-38.0; Atkinson and Ets 28.0; Haden and Orr 30.3; Draper 44.0. Urea nitrogen: Bang 17.0-27.0; Atkinson and Ets 17.9; Haden and Orr 11.1; Marshall and Davis 12.5-13.0; Austin and Stillman and Van Slyke 9.2-10.9; Hammond 12.1; Draper 18.0. Amino-acid nitrogen: Van Slyke and Meyer 3.0-5.0; Gyorgy and Zunz 4.0-5.0; Okada and Hayashi 7.3; Bock 7.5. Uric acid: Haden and Orr 1.5; Draper 0.7. Creatine: Mathews 1.54 for young animals and 2.92 for adult animals. Creatinine: Haden and Orr 1.2-2. Sugar: Fujii 80-110; Haden and Orr 76; Atkinson and Ets 103; Draper 125. Chlorides: Hastings, Murray and Murray 304.0-306.5; Haden and Orr 277.

Morgulis and Edwards concluded, from their study of the chemistry of the blood during fasting, that the non-protein nitrogen and urea nitrogen of the blood usually increased at an early stage of fasting and remained at a more or less fixed level until the extreme stage was reached, when a new and much greater increase occurred. The blood uric acid increased progressively during fasting. The creatinine remained constant, but the creatine, following a diminution which may have occurred during an early stage, rose rapidly in the most advanced stage of fasting. The sugar usually diminished in the course of the first of the fasting period, but increased again during the later stages. When the animals were fed following a protracted fast, both the non-protein nitrogen and urea nitrogen and the uric acid and creatine, decreased rapidly even in the first few days of re-alimentation.

Pucher (129) found that in new born puppies the average urea nitrogen was 20.7 milligrams and sugar 110 milligrams per 100 c.c. of blood. He stated that new born puppies showed a marked urea nitrogen retention after fasting, whereas six weeks old animals showed much less nitrogen retention under similar experimental conditions.

Benedict (14) in 1916 reported the observation that the Dalmation breed of coach hounds exhibited a marked peculiarity in its purine metabolism, to the extent that it excreted large amounts of uric acid, even when on a purine-free diet. This peculiarity was exhibited by four Dalmations examined, but a fifth, "obviously not of very pure breed," did not eliminate much uric acid. He states "that a single species of dog should exhibit this characteristic is remarkable, especially in view of the fact that the only other mammals that excrete uric acid in any considerable amounts are man and the anthropoid apes. The Dalmation coach hound, however, differs also from man and the anthropoids in that it excretes allantoin in considerable amounts, i. e., from one-half to two-thirds as much allantoin as uric acid. Hence the Dalmation is unique in respect to purine metabolism."

With one dog of this breed, weighing about 10 kilograms, on a diet containing only 2.03 grams of total nitrogen, there was an excretion of 0.154 grams of uric acid nitrogen and 0.073 grams of allantoin nitrogen, with a total nitrogen output of 5.4 grams. Further, Benedict found that the uric acid showed no gain when the nitrogenous food was increased four-fold, although the output of allantoin increased as the nitrogen intake was augmented.

For a period of nearly a year a dog of this breed was kept upon a purine-free diet and during nearly all of this time the uric acid elimination was determined daily. The total amount of uric acid eliminated during this



period was more than 100 grams, while the animal maintained a constant body weight. He concluded that not ten per cent of this quantity of uric acid could have come from the purine of the animal's tissues. As a result of this study, Benedict concluded that the adult mammal could synthesize purine from non-purine material.

Some of Benedict's observations were corroborated by Wells (154), who observed a Dalmation coach hound that excreted large quantities of uric acid. The liver of the same dog possessed the power of destroying uric acid *in vitro*, which indicated that the presence of uric acid in the urine of the Dalmation did not depend upon the absence of uricase in the tissues. The kidneys did not show any uricolytic activity. He also found that while the liver of the animal deaminized both adenine and guanine, neither the liver nor the spleen could convert xanthine into uric acid.

Schwarz and Hamp (139) found that the blood sugar value in an individual dog remained constant twelve hours after the taking of food, if the food remained the same. Hunger was accompanied by a decrease in the blood sugar in a well nourished dog. The blood sugar value mounted in a short time after the food was taken. The blood sugar reached its high point in the second and third hour. About the twelfth hour it reached its normal value. The maximum values were between 28 and 35 per cent above the normal value.

Abderhalden (1) gave 3.5 milligrams of inorganic phosphorus and 8.1 milligrams of calcium per 100 c.c. of serum as values for the dog.

*Cats.* Folin and Denis (58) reported determinations of uric acid, non-protein nitrogen and urea nitrogen in the blood of six cats. The uric acid value was 0.2 milligrams per 100 grams of blood in each case. The non-protein nitrogen ranged from 31 to 67 milligrams, with an average of 52.6. Urea nitrogen ranged from 20 to 37 milligrams, with an average of 30.3 milligrams per 100 grams of blood. These writers (57) previously reported 1.2 milligrams of creatinine and 8 milligrams of creatine plus creatinine per 100 c.c. for the blood of the cat.

Abderhalden (1) gave 3.1 milligrams of inorganic phosphorus and 7.8 milligrams of calcium per 100 c.c. of serum as values for the cat.

*Rats.* In 1913 Folin and Morris (60) published data on the uric acid, non-protein nitrogen and urea content of rat blood. In their work they used the mixed blood of six normal rats and found 2 milligrams of uric acid, 38 milligrams of non-protein nitrogen and 22 milligrams of urea per 100 c.c. Voegtlin, Dunn and Thompson (147) in 1924 found an average of 113 milligrams of sugar per 100 c.c. of rat blood.

Anderson, Honeywell, Santy and Pederson (4) found that in most respects rat blood was quite similar to human blood. The most noticeable exception was in the case of non-protein nitrogen where the average value was considerably above that for human blood. Their averages were as follows: sugar 122 milligrams, total non-protein nitrogen 45.2 milligrams, creatinine 1.27 milligrams, creatine plus creatinine 6.42 milligrams, urea nitrogen 15.6 milligrams, and uric acid 1.86 milligrams per 100 c.c. of blood.

Chanutin and Silvette (35) found in a group of seven control rats, the blood constituents averaged as follows: non-protein nitrogen 43 milligrams, with variations between 37 and 51 milligrams; sugar 136 milligrams, varying between 115 and 154; total creatinine 5.7 milligrams, with variations



between a minimum of 5.1 and a maximum of 6.7; preformed creatine averaged 1.4 milligrams per 100 c.c. and ranged between 1.1 and 2.1 milligrams per 100 c.c. of blood. In addition, Chanutin and Silvette studied the effect of fasting on these constituents and concluded that there was little change in the non-protein nitrogen or creatine and creatinine. The blood sugar values fell in the early stages of fasting this condition was followed by a rise above normal with further progress of the fast. The feeding of creatine caused the blood creatine to markedly increase, but did not cause a decrease in the blood sugar as Hill (87) encountered in the dog and man.

*Guinea Pigs* Teich (146) reported that the calcium content in the blood of normal guinea pigs averaged 0.073 grams per 1,000 c.c. in seventeen animals with a range from 0.060 to 0.130 grams per 1,000 c.c. of blood.

*Rabbits.* Culhane (40) reported variations in serum calcium in rabbits, with values from 14.31 to 17.12 milligrams, with an average of 15.47 milligrams per 100 c.c. for six animals studied. He stated that insulin caused no rise in the serum calcium. The feeding of cabbage caused an immediate rise except when the initial value could be regarded as exceptionally high.

Brown (28) reported the results of five hundred ninety-seven determinations of calcium and phosphorus in thirty-six rabbits. The average value for calcium was 15.6 milligrams and for inorganic phosphorus 4.51 milligrams per 100 c.c. of serum. He stated that the mean values for calcium were higher than most of those recorded in the literature and that the values for inorganic phosphorus were perhaps lower. It was found that both calcium and inorganic phosphorus showed wide ranges of variation with a tendency to vary in opposite directions. He found that the inorganic phosphorus content of the blood of normal rabbits was higher than in rabbits that had been kept for some time in the laboratory.

Harnes (79) made determinations of calcium and inorganic phosphorus on a series of rabbits recently received from the dealer and found that for the eighty animals examined, calcium varied from 14.5 to 18.5 milligrams and inorganic phosphorus from 4.96 to 6.82 milligrams per 100 c.c. of serum. He also found that over the same period of time, rabbits living in the laboratory maintained a higher level of calcium than those living out of doors. The inorganic phosphorus showed a decrease.

Allers and Bondi (2) doubled the calcium content in the blood of rabbits by feeding large doses of hydrochloric acid.

Folin and Denis (53) reported observations on the blood of six rabbits. The average values per 100 grams of blood were as follows: uric acid 0.05 milligrams; non-protein nitrogen 31 milligrams; urea nitrogen 13 milligrams. They (57) reported also values of 1 milligram per 100 c.c. for creatinine and 10 milligrams per 100 c.c. for creatine and creatinine in the blood of the rabbit.

Watkins and Smith (153) reported figures for blood constituents determined in three rabbits as follows: in a normal doe the sugar value was 124 milligrams, non-protein nitrogen 20.3 milligrams, and urea nitrogen 11.4 milligrams per 100 c.c. of blood; for a seventeen days pregnant doe the sugar value was 110 milligrams, non-protein nitrogen 21.7 milligrams, and urea nitrogen 12.5 milligrams per 100 c.c.; for a lactating doe the sugar value was 113 milligrams, non-protein nitrogen 25.8 milligrams, and urea

nitrogen 18 milligrams per 100 c.c. Adrenalin caused a rise in blood sugar, due to a decreased rate of oxidation. There was also a rise in urea nitrogen which occurred later with some increase in the urinary urea.

Ruggeri (135) found a hyperglycemia in castrated rabbits following the injection of diuretics. In some cases the sugar level rose to 2.8 and 3.2 grams per 1,000 c.c. of blood the second hour after injection. The blood sugar levels were normally high.

Abderhalden (1) gave the values of 2.8 milligrams of inorganic phosphorus and 8.3 milligrams of calcium per 100 c.c. of plasma for the rabbit.

### *The Blood of Farm Animals*

The study of the chemical composition of the blood of farm animals has received but slight consideration from investigators.

*Horses.* Scheunert and Pelchrzim (137) reported the results of observations of blood constituents on seven normal horses ranging from 4 to 15 years of age. The blood sugar varied between 76.9 and 200 milligrams, with an average of 138.5 milligrams per 100 c.c.; the rest nitrogen (probably total non-protein nitrogen), between 21.4 and 30 milligrams, with an average of 29 milligrams per 100 c.c.; the urea nitrogen between 7.5 and 15 milligrams, with an average of 9.64 milligrams per 100 c.c.; the creatinine between 1.2 and 1.7 milligrams, with an average of 1.27 milligrams per 100 c.c.; the creatine plus creatinine between 5.0 and 6.6 milligrams, with an average of 5.9 milligrams per 100 c.c. of blood.

Scheunert and Bartsch (136) reported that the normal or average daily work did not affect the content of horses' blood in sugar, rest nitrogen, ammonia nitrogen, creatinine and creatine plus creatinine. The carbon dioxide combining power as a rule increased.

Holt and Reynolds (89) reported observations on the blood constituents of a large number of normal horses, with the following results: The average for hemoglobin from 136 cases was 88.2 per cent, with a variation between 70 and 98 per cent. The average of 141 determinations for blood sugar was 0.109 per cent, with a variation between 0.078 and 0.37 per cent. Urea nitrogen averaged 14.81 milligrams per 100 c.c. of blood for 141 determinations and varied between 7.5 and 25.0 milligrams. Non-protein nitrogen averaged 25.82 milligrams per 100 c.c. of blood for 141 determinations and varied between 16.5 and 42.58 milligrams. Uric acid averaged 2.76 milligrams per 100 c.c. of blood for 138 determinations and varied between 1.76 and 6.66 milligrams. Creatinine averaged 1.4 milligrams per 100 c.c. of blood and varied between 0.9 and 3 milligrams.

Holt and Reynolds continued observations on the blood of horses in eight cases of disease, with the following results: One case of laminitis showed 0.136 per cent of blood sugar, 12.0 milligrams of urea nitrogen, 23.62 milligrams of non-protein nitrogen, 3.84 milligrams of uric acid and 2.1 milligrams creatinine per 100 c.c. of blood. Another case of laminitis showed 65 per cent of hemoglobin, 0.115 per cent of blood sugar, 16.65 milligrams of urea nitrogen, 23.1 milligrams of non-protein nitrogen, 3.91 milligrams of uric acid and 2.1 milligrams of creatinine per 100 c.c. of blood. A case of periodic ophthalmia showed 81 per cent of hemoglobin, 0.136 per cent of sugar, 18.68 milligrams of urea nitrogen, 20.0 milligrams of non-protein nitrogen, 6.25 milligrams of uric acid and 2.8 milligrams of

creatinine per 100 c.c. of blood. A case of ophthalmia showed 83 per cent of hemoglobin, 0.1 per cent of sugar, 12.5 milligrams of urea nitrogen, 17.14 milligrams of non-protein nitrogen, 5.0 milligrams of uric acid and 3.0 milligrams of creatinine per 100 c.c. of blood. A case of strangles showed 84 per cent of hemoglobin, 0.093 per cent of sugar, 12.0 milligrams of urea nitrogen, 27.27 milligrams of non-protein nitrogen, 6.0 milligrams of uric acid and 1.1 milligrams of creatinine per 100 c.c. of blood. A case of dermatitis showed 80 per cent of hemoglobin, 0.084 per cent of sugar, 12.5 milligrams of urea nitrogen, 19.98 milligrams of non-protein nitrogen, 2.0 milligrams of uric acid and 1.0 milligram of creatinine per 100 c.c. of blood. A case of rhinitis showed 88 per cent of hemoglobin, 0.07 per cent of sugar, 8.82 milligrams of urea nitrogen, 30.0 milligrams of non-protein nitrogen, 2.35 milligrams of uric acid and 1.7 milligrams of creatinine per 100 c.c. of blood. A case of chronic articular rheumatism showed 82 per cent hemoglobin, 0.10 per cent of sugar, 10.0 milligrams of urea nitrogen, 20.0 milligrams of non-protein nitrogen, 2.66 milligrams of uric acid and 1.2 milligrams of creatinine per 100 c.c. of blood.

The values of these constituents in the diseased animals are within the range of variation for normal animals.

Hayden and Fish (82) report 102.9 milligrams of sugar per 100 c.c. of blood for the horse, 18.7 milligrams of urea (undoubtedly urea nitrogen). Three readings of horse blood averaged 0.69 milligrams of uric acid per 100 c.c. of blood, total non-protein nitrogen 33.9 milligrams per 100 c.c., and preformed creatinine 1.81 milligrams per 100 c.c. of blood. In this report they state that there is some evidence to indicate that seasonal conditions may modify the results.

Clark (38) gave figures for the calcium content in horse plasma determined by direct precipitation as calcium oxalate and titrated against 0.01 N potassium permanganate. These figures recalculated in terms of milligrams ranged from 9.9 to 12.3 milligrams, with an average of 11.26 per 100 c.c. of plasma.

Rona and Takahashi (134) found that under ordinary circumstances horse serum contained about 12 milligrams of calcium per 100 grams of blood.

Folin and Denis (58) reported 0.05 milligrams of uric acid, 54 milligrams of non-protein nitrogen, and 28 milligrams of urea nitrogen per 100 grams of blood for the horse.

Brocq-Rosseau, Roussel and Gallot (28) estimated the urea in the blood of 33 horses. The average value was 0.314 grams per liter, ranging from 0.165 to 0.453 grams. Determinations were also made before and after the injection of six liters of fluid, and they found that the content of urea in the blood was quite independent of the amount of fluid or concentration of the blood.

Schwarz (138) found the average value for blood sugar in the horse ranged between 0.062 and 0.120 per cent. In 26 per cent of the cases the values were between 0.062 and 0.082 per cent. In 46 per cent of the cases the values were between 0.083 and 0.102 per cent. In 27 per cent of the cases the values were between 0.103 and 0.120 per cent. He found that sex had no influence.

Abderhalden (1) reported 7.9 milligrams of calcium and 3.3 milligrams of inorganic phosphorus per 100 c.c. of serum for the horse.



*Cattle.* Scheunert and Pelchrzim (137) reported the results of determinations of the blood constituents of ten cows in different stages of lactation and pregnancy. The blood sugar varied between 62.5 and 100 milligrams, with an average of 76.65; the rest nitrogen (non-protein nitrogen) varied between 26.3 and 39.4 milligrams, with an average of 28.74; the urea nitrogen values were between 10.5 and 23.7 milligrams, with an average of 18.09 milligrams per 100 c.c.; the creatinine values were between 1.4 and 1.8 milligrams, with an average of 1.61 milligrams per 100 c.c.; and the creatine plus creatinine varied between 5.7 and 10.4 milligrams, with an average of 7.71 milligrams per 100 c.c. of blood. They stated that pregnancy and lactation in its various stages had no significant influence on the blood constituents.

Hayden and Fish (82) studied the composition of the blood of cows. Sixty-eight sugar determinations were made. The lowest reading was 30 milligrams, the highest was 70, and the average was 46.52. The average was slightly lower for dry cows than for milch cows.

Sixty-seven urea (nitrogen) determinations were made upon the blood of cows. The values were between 5.3 and 27 milligrams, with an average of 12.21 milligrams per 100 c.c. Seventy-three determinations of uric acid in the blood of cows were made by the Folin direct method. These averaged 2.04 milligrams per 100 c.c. of blood. Thirty-three readings of uric acid were made by the isolation method of Folin, which gave an average of 0.623 milligrams per 100 c.c. of blood.

A range of values between 25 and 35 milligrams of total non-protein nitrogen per 100 c.c. was given by these authors for the blood of the cow. Preformed creatinine gave readings similar to those of human blood, 1 to 2 milligrams per 100 c.c.

Anderson, Gayley and Pratt (3) reported analyses of 59 samples of blood taken from the dairy herd of the Pennsylvania State College. The animals included calves and adults of various ages and breeds. The animals received the regular ration used for the dairy herd. The blood samples were taken during the late summer, fall and winter and no attempt was made to study seasonal variations.

Hemoglobin, expressed as percentage normal, gave values ranging from 70.3 to 121.7 per cent. The average value was 92.9 per cent for 29 analyses. The hemoglobin content appeared to be higher in animals less than one month of age.

Their figures for non-protein nitrogen from 50 determinations ranged from 20.67 to 42.14 milligrams, with an average of 30.07 milligrams per 100 c.c. of blood. Fifty estimations for urea nitrogen gave an average of 12.94 milligrams per 100 c.c. of blood, with minimum and maximum values of 4.4 milligrams and 21.64 milligrams, respectively.

Fifty-nine determinations of uric acid, using the direct method of Benedict, gave an average of 2.08 milligrams per 100 c.c. of blood, with values ranging from 1.5 to 3.22 milligrams.

Anderson and his co-workers found an average of 4.3 milligrams of creatine per 100 c.c. of blood, with values ranging from 2.49 to 7.78. From fifty-nine determinations for creatinine they obtained an average of 1.42 milligrams per 100 c.c., with values ranging from 1.19 to 1.94. The sugar values, for fifty-eight determinations, ranged between 43.2 and 14.2 milligrams,



with an average of 84.1 milligrams per 100 c.c. of blood. The blood sugar values decreased as the age of the animal increased.

From twenty analyses for inorganic phosphorus Anderson, Gayley and Pratt obtained an average of 4.46 milligrams per 100 c.c. of serum, with values ranging from 3.09 to 6.17 milligrams per 100 c.c. Their results for calcium gave an average of 12.63 milligrams per 100 c.c. of serum for fifty-five analyses, ranging from 9.96 to 16.18 milligrams per 100 c.c. They noted no significant differences in the calcium content of young and mature animals.

Folin and Denis (57) reported 2 milligrams of creatinine and 11 milligrams of creatine plus creatinine per 100 c.c. of beef blood. Greenwald and McGuire (68), using Folin's method, obtained an average of 2.57 milligrams of creatinine for oxalated beef blood and 9.81 milligrams of creatine plus creatinine. By using another method they obtained a value of 1.61 milligrams for creatinine and 2.8 milligrams for creatine.

Robinson and Huffman (131) reported the analyses of more than one hundred samples of blood from normal mature cattle. The inorganic phosphorus ranged from 3 to 8.99 milligrams per 100 c.c. of serum, with an average of 5.87. The maximum 24 hour variation was 1.87 milligrams. Calcium values ranged from 7.7 to 14.7 milligrams, with an average of 11 milligrams per 100 c.c. of serum. The maximum variation for 24 hours was 2 milligrams.

Hart and co-authors (80) reported that the calcium content of the blood of milking cows was high during a period of feeding timothy hay when the calcium losses were greatest. The calcium content was between 20 and 25 milligrams per 100 c.c. of serum. When alfalfa was fed the calcium dropped to approximately 10 milligrams per 100 c.c. of serum, but rose to 16 milligrams during the period that timothy hay and bone meal were fed. Inorganic phosphorus was low, 2.57 milligrams per 100 c.c. of serum, during the timothy hay period, but was considerably higher, with an average of 4.62 milligrams, in alfalfa and timothy hay plus bone meal periods.

Meigs, Blatherwick and Cary (112) reported 47 determinations of inorganic phosphorus on cows which averaged 5.3 milligrams per 100 c.c. of serum and ranged from 3.8 to 8.8 milligrams per 100 c.c. They reported an average of 9.6 milligrams of calcium per 100 c.c. of serum in pregnant cows not lactating and 9.8 milligrams in lactating cows not pregnant.

Folin and Denis (58) reported determinations of constituents of mixed beef blood giving the following values: uric acid 0.2 milligrams, non-protein nitrogen 24 milligrams, and urea nitrogen 14 milligrams per 100 grams of blood. They (57) reported 2 milligrams of creatinine and 11 milligrams of creatine plus creatinine in beef blood.

Benedict (13), employing the method described by him (12), found an average value of 0.5 milligrams of uric acid per 100 c.c. of defibrinated fresh ox blood, based upon the analyses of seven samples of mixed blood. The highest value obtained was 0.67 and the lowest 0.41 milligram. He pointed out that uric acid determined by this method was "free" uric acid and that this was only a small fraction of the uric acid present. The remainder existed in a "combined form" from which it was easily set free by hydrolysis with acid. Davis and Benedict (42) have shown that the uric acid is combined with a pentose. After hydrolysis of a protein-free blood filtrate, Benedict (13) obtained values as high as 6.7 milligrams per 100 c.c. of blood.

Newton and Davis (123) showed that the combined uric acid in beef blood was contained in the erythrocytes. They found also evidences of the presence of combined uric acid in the blood of the human, horse, sheep, pig, dog and chicken. The quantity in beef blood far exceeded that in any other animal blood studied. Next in quantity would appear to be that of human blood.

Hewitt (86) found that the blood sugar concentration was decidedly higher in heifers and non-lactating cows than in lactating cows. Evidence was found that extremely high blood sugar levels may have been correlated with oestrus in heifers.

Schwarz (138) found that the average value of blood sugar in normal cattle was 0.082 per cent, with values between 0.044 and 0.177 per cent.

Awdejewa, Prowatorowa and Sawitsch (6) found that the blood sugar in nursing cows had a low level. Carbohydrate feeds did not increase the blood sugar. The variations of the blood sugar content in the same animal on different days were as significant as the variations between various animals on the same day. The low sugar content and the variations were not dependent upon milking.

Richter (130) found that if the feeding was regular and constant, and the blood always taken in the same manner, the blood sugar level in milch cows showed no daily variation. The nearly absolute value which in general was obtained in milch cows was lower than in dry cows.

Richter found in the bovine no increase in blood sugar value after feeding, as reported by Schwarz and Hemp (139) in dogs. The reason was that under normal conditions the stomach was never empty. The taking of food had no influence on the absorption from the intestine as long as food was contained at the normal level in the forestomachs.

Aberhalden (1) gave 2.7 milligrams as the value for inorganic phosphorus and 7.9 milligrams for calcium per 100 c.c. of serum for the ox.

Horveth and Little (90) studied the blood composition in bronchopneumonia of cows. They found the non-protein nitrogen higher when the process in the lungs was not completely healed, and reached a normal level during recovery. They stated that high non-protein nitrogen and creatinine were symptoms of secondary kidney involvement.

*Sheep.* Scheunert and Pelchrzim (137) reported the following values for certain constituents in the blood of sheep. Sugar ranged from 50.9 to 88.9 milligrams per 100 c.c. of blood, with an average of 65.6 milligrams per 100 c.c. for sixteen determinations. Rest nitrogen averaged 28.5 milligrams, ranging from 21.8 to 42.9 milligrams per 100 c.c. for sixteen determinations. Urea nitrogen averaged 11.8 milligrams per 100 c.c. of blood for sixteen determinations with a range from 7.5 to 23.1 milligrams. Five determinations of creatinine averaged 1.26, varying between 1.2 and 1.3 milligrams. Five determinations of creatine plus creatinine averaged 4.14 milligrams, ranging from 3.8 to 5.3 milligrams per 100 c.c. of blood.

Folin and Denis (58) reported 0.05 milligrams of uric acid, 31 milligrams of non-protein nitrogen, and 13 milligrams of urea nitrogen per 100 c.c. in the mixed blood of sheep. They (57) reported 1.2 milligrams of creatinine and 9 milligrams of creatine plus creatinine per 100 c.c. in blood of sheep.

Aberhalden (1) gave values of 8.4 milligrams of calcium and 3.2 milligrams of inorganic phosphorus per 100 c.c. of serum for sheep.

*Swine.* Hayden and Tubangui (83) made determinations on the blood of swine, some of the blood samples having been collected at the time of slaughter and some from immunized pigs by tail bleeding.

The total non-protein nitrogen averaged 35.0 milligrams per 100 c.c. of blood for fourteen determinations, with variations between 30.6 and 38.3 milligrams. Urea nitrogen averaged 19.6 milligrams per 100 c.c. of blood for fourteen determinations and ranged between 15.6 and 27.3 milligrams. The uric acid readings averaged 2.06 milligrams per 100 c.c. of blood for fourteen determinations, with minimum and maximum readings of 1.73 and 2.5 milligrams respectively. Creatinine averaged 1.42 milligrams per 100 c.c. of blood for twenty determinations, ranging from 1.14 to 2.00 milligrams. Sugar averaged 90.1 milligrams per 100 c.c. of blood for twenty determinations and ranged between 76 and 109 milligrams.

Scheunert and Pelchrzim (137) reported the determinations of certain blood constituents of a group of swine consisting of five males and ten females. The average weight for the group was 251 pounds. The averages for the blood constituents in milligrams per 100 c.c. of blood were as follows: sugar 92.38, rest nitrogen 31.1, urea nitrogen 15.38, creatinine 1.78, and creatine plus creatinine 6.32.

Scheunert and Pelchrzim (137) found no uric acid in the blood of swine.

Folin and Denis (58) gave the following values for uric acid, total non-protein nitrogen and urea nitrogen per 100 grams of mixed blood of swine: uric acid 0.05 milligram, non-protein nitrogen 32 milligrams, and urea nitrogen 14 milligrams. They (57) reported 1.3 milligrams of creatinine and 9 milligrams of creatine plus creatinine per 100 c.c. of the blood of the pig.

Greenwald and McGuire (68), using Folin's method, reported two determinations of creatinine in pig's blood of 4.18 and 2.8 milligrams per 100 c.c. of blood, respectively, and for creatine plus creatinine 6.44 and 9.1 milligrams per 100 c.c. of blood, respectively.

Roderick and Schalk (132) made determinations of non-protein nitrogen, urea (nitrogen) and sugar in the blood of apparently normal hogs. Eight determinations of non-protein nitrogen gave an average value of 41.5 milligrams per 100 c.c. of blood, ranging between 28 and 49.9 milligrams. Urea (nitrogen) ranged between 11 and 23.4 milligrams per 100 c.c. of blood, with an average value of 17.3 milligrams for twelve determinations. Sugar ranged between 70 and 135 milligrams, with an average of 85.7 milligrams per 100 c.c. of blood for twelve determinations.

Aberhalden (1) gave values of 2.3 milligrams for inorganic phosphorus and 8.7 milligrams for calcium per 100 c.c. of serum for swine.

Buell (30), in studying the effect of hemorrhage on the alkaline reserve in the blood of pigs, noted that the total nitrogen content of the blood always fell immediately after hemorrhage. There was a distinct tendency for the urea nitrogen and the non-protein nitrogen to rise.

Carlson and Drennan (34) stated that the normal pig had a lower tolerance for dextrose, bread or cooked starch, given by mouth, than any species studied. A normal pig weighing 8 to 10 kilos showed marked alimentary glycosuria when given 2.5 grams of dextrose, and on an empty stomach the lack of tolerance seemed to border on the pathological. This lack of tolerance was due to failure of adjustment of the rate of absorption



to the rate of fixation or storage of sugar. They stated that this condition in the pig must lead to a waste of calories in proportion to the quantity of bread or cooked starch fed.

Shope (142) found that the distribution of sugar between corpuscles and plasma varied with the species. Using a limited number of animals in each species he found the following average values for the percentage of total sugar in the corpuscles: swine 24 per cent, guinea pigs 48 per cent, cattle 30 per cent, rabbits 20 per cent, man 49 per cent.

Hayden and Tubangui (83) compared the average of their results for the determination of blood constituents in milligrams per 100 c.c. of blood with the results of Folin and Denis, Benedict, Greenwald, and Greenwald and McGuire. Folin and Denis obtained the following averages: for cattle blood, total non-protein nitrogen 24, urea 14, uric acid 0.20, creatinine 2.0 and sugar 96; for horse blood, total non-protein nitrogen 54, urea 28, uric acid 0.05 and sugar 106; for sheep blood, total non-protein nitrogen 28, urea 13, uric acid 0.05, creatinine 1.2, sugar 105; for swine blood, total non-protein nitrogen 32, urea 14, uric acid 0.05, creatinine 1.4 and sugar 97.1. Greenwald obtained 32 milligrams of total non-protein nitrogen as the average for cattle blood and 27 milligrams of total non-protein nitrogen for sheep blood. Benedict obtained 0.5 milligrams of uric acid in cattle blood. Greenwald and McGuire obtained 1.9 milligrams of creatinine as the average for cattle blood, 1.08 for sheep blood and 2.2 for swine blood. Hayden and Tubangui obtained the following averages: for cattle blood, total non-protein nitrogen 51, urea 18.8, uric acid 2.12, creatinine 1.84 and sugar 96; for horse blood, total non-protein nitrogen 34, urea 17.8, uric acid 2.45, creatinine 1.8 and sugar 106; for sheep blood, total non-protein nitrogen 32, urea 17.8, uric acid 1.82, creatinine 1.26 and sugar 105; for swine blood, total non-protein nitrogen 19.6, uric acid 2.06, creatinine 1.42 and sugar 97.1.

Scheunert and Pelchrzim (137) have arranged in milligrams per 100 c.c. the result of their determinations of certain blood constituents on various animals, including results obtained from human blood by various investigators. Human blood gave the following values: sugar 77-119, rest nitrogen 24-43, urea nitrogen 10-22, total creatinine 5.3-6.7, creatinine 1.2-2.5, uric acid 1.4-2. Scheunert and Pelchrzim made determinations on the blood of several animals. Seven determinations on two dogs gave the following values: sugar 89-111, rest nitrogen 22.2-32.4, urea nitrogen 11.5-16.7, total creatinine 3.8-4.1, creatinine 1.2-1.5. Sixteen determinations on four sheep gave the following values: sugar 51.1-89.9, rest nitrogen 21.8-42.9, urea nitrogen 7.5-23.1, total creatinine 3.9-5.3, creatinine 1.2-1.3. Ten determinations on five head of cattle gave the following values: sugar 62.5-100, rest nitrogen 23.8-39.4, urea nitrogen 10.5-23.7, total creatinine 5.7-10.5, creatinine 1.4-1.7. Seven determinations on seven horses gave the following values: sugar 83.3-200, rest nitrogen 21.4-30, urea nitrogen 7.5-15, total creatinine 5-6.6, creatinine 1.2-1.7. Fifteen determinations on fifteen head of swine gave the following values: sugar 80-106.7, rest nitrogen 25-40, urea nitrogen 10-18.8, total creatinine 5.1-8.7, creatinine 1.7-1.9.

*The Blood of Cholera-infected Swine.* The only reference that could be found pertaining to the constituents of the blood in hog cholera, was that of Roderick and Schalk (132) in which they record the results of a series of analyses on the blood of apparently normal and of cholera-infected



hogs. The blood was secured usually by aspiration from the heart and the determinations were made according to the methods of Folin.

They stated that the blood of cholera-infected pigs drawn at various stages of the disease was often normal so far as the non-protein nitrogen was concerned. Other samples of blood showed moderate evidences of nitrogenous retention. It did not appear that any particular stage of the disease was more prone to show retention than others. No abnormal amounts of blood sugar were found.

Roderick and Schalk divided the cholera-infected bloods into two groups, one group which showed no retention of nitrogen and one group which showed appreciable nitrogen retention.

In the group that showed no retention of nitrogen, fifteen determinations gave an average value of 39.4 milligrams of non-protein nitrogen per 100 c.c. of blood, with values ranging from 30 to 48 milligrams per 100 c.c. Sixteen determinations of urea nitrogen ranged from 13 to 25.4, with an average value of 16.5 milligrams per 100 c.c. of blood. Fifteen determinations of sugar gave an average of 88.4 milligrams, with values ranging from 71 to 122 milligrams per 100 c.c. of blood.

In the group that showed appreciable retention of nitrogen consisting of ten analyses, the non-protein nitrogen showed an average of 65.5 milligrams, with values between 51 and 88 milligrams per 100 c.c. of blood. Urea nitrogen showed an average of 33.9 milligrams and ranged between 19.3 and 55.0 milligrams per 100 c.c. of blood. Sugar averaged 82.7 milligrams, ranging between 52 and 112 milligrams per 100 c.c. of blood.

These writers (132) state that, "The degree of retention occasionally encountered in this work does not compare with that in uremia in man. Glomerulonephritis and contracted kidneys are rare in animals. The injury to the kidney in hog cholera usually is a nephrosis and while acutely swollen kidneys are occasionally encountered they are not common. The retention of the nitrogenous metabolites in nephrosis is not a striking characteristic of that type of kidney injury. The hemorrhages in the hog cholera kidney are often interstitial although the presence of blood and hemoglobin in the urine are referable to glomerular injury and hemorrhage. The fact that the tubular epithelium is mainly involved suggests that the virus of hog cholera is toxic in the nature of its injury."

They concluded from this phase of their studies that the carbohydrate and nitrogenous metabolism in hog cholera showed little alteration. The degree of nitrogen retention found at times was explained by the nephrosis which seemed to be the chief form of kidney injury in addition to the hemorrhage.

## MATERIALS AND MEHTODS

### ANIMALS USED

The normal blood samples were obtained from pigs of various sources and localities. It was necessary to depend upon several departments of the University of Minnesota and Iowa State College as well as the Experiment Station of the United States Bureau of Animal Industry and commercial institutions for the material used in these studies. It was not possible to adequately control numerous factors that probably influenced the concentration of certain of these constituents in the blood of swine. However, the large number of animals studied would tend to offset the lack of

control. The work was begun a number of years ago as the accumulation of data was slow.

### *Minnesota Swine*

Thirty-five of the blood samples studied were secured from pigs which were purchased from the stock yards at South Saint Paul, and brought to the University Farm to be used in other experimental work. Opportunity was given to collect blood from these animals before they were subjected to any experimental procedure. Four blood samples were obtained from animals that were slaughtered at the Midway abattoir in Saint Paul. Five blood samples were obtained from pigs slaughtered at one of the packing houses in South Saint Paul. Three animals were loaned to the Veterinary Division for this study by the Animal Husbandry Division at University Farm. Four chemical analyses were made on the blood of each of these animals.

### *Iowa Swine*

Blood samples were obtained from four pigs owned by the Department of Veterinary Research, Iowa State College. Fifty-four blood samples were obtained from pigs that were slaughtered at the Iowa State College abattoir. These pigs were raised on farms near Ames, Iowa. One blood sample was obtained from a female pig two years of age which was owned by the Department of Veterinary Research and slaughtered at the Iowa State College abattoir.

The weights of these pigs varied. In a number of cases accurate weights were obtained, while in others the weights were estimated. The pigs ranged in weight from 50 to 300 pounds. One female two years of age weighed 415 pounds. It was impossible to obtain accurate data in regard to the age of a great number of these animals.

The samples of cholera-infected blood were obtained at the time of slaughter from pigs that had been injected with hog cholera virus six to twelve days previous. These animals weighed between 40 and 90 pounds each. After the blood was drawn the animals were autopsied to confirm the diagnosis of hog cholera. Eight of the cholera-infected blood samples were obtained from pigs that belonged to the Department of Veterinary Research, Iowa State College. Nineteen of the blood samples were taken from animals that were the property of the Experiment Station of the United States Bureau of Animal Industry located at Ames, Iowa. Twenty-seven of the blood samples were obtained from pigs that belonged to the plant of the Fort Dodge Serum Company of Fort Dodge, Iowa.

### BLOOD SAMPLES

The technique for the methods used in this paper was acquired chiefly in the laboratories of the Department of Physiology of the Medical School, University of Minnesota, using the blood of various laboratory animals. The details for the procedures of the various analyses are described in standard texts dealing with blood analysis.

The data reported in this paper were accumulated over a considerable period of time. Blood samples were obtained from forty-two of the normal

animals by tail bleeding. The remaining blood samples were obtained when the animals were killed by throat bleeding. Usually between 50 and 70 c.c. of blood were collected from each animal. Sixty milligrams of sodium citrate per 10 c.c. of blood were used to prevent coagulation. It was found after several trials that swine blood required about three times as much anticoagulant as did bovine or equine blood. Sodium citrate was chosen as the anticoagulant instead of potassium oxalate, because serum was obtained from the same samples for calcium determinations. Two cubic centimeters of 20 per cent solution of sodium citrate were introduced into each collecting tube and evaporated to dryness. The evaporating solution spattered, and in drying formed a coating of citrate on the wall of the tube.

In the chemical examination of blood, potassium oxalate is most frequently used as an anticoagulant. It is the general impression that sodium citrate should not be used for the reason that it supposedly interferes with deproteinization, especially when tungstic acid is used as the protein precipitant. It is further claimed that the citrate interferes with the determination of uric acid (81, p. 367).

Leiboff and Witchell (106) studied the effects of potassium oxalate and of sodium citrate upon some of the chemical constituents of the blood. They reported no difficulty in obtaining clear filtrates in the presence of citrate, using tungstic acid as the protein precipitant. In this respect the citrate and the oxalate behaved alike.

They made the following determinations on blood filtrates; non-protein nitrogen by the method of Koch and McMeekin (102); urea nitrogen by the method of Folin and Denis; uric acid by the method of Folin (52, 49); creatinine by the method of Folin (52); sugar by the method of Folin and Wu (62).

The citrate not only did not interfere with the determination of uric acid, but was superior to the oxalate, as some of the tubes containing oxalate became very cloudy on adding the reagents and little color developed. All the tubes containing citrate, even those containing the largest amounts, remained clear and developed a good color.

As for the other constituents determined, in the presence of excess citrate the non-protein fraction was somewhat increased, while the amount of sugar was somewhat decreased. Practically, this was of no significance since the amount of citrate necessary to produce such faulty results was far in excess of the amount necessary to prevent coagulation.

It is obvious that oxalated blood cannot be used for the determination of calcium. Sodium citrate forms soluble non-ionizable calcium citrate and is the ideal anticoagulant in making calcium determinations. Portions of a number of the blood samples were used for determining the volume percentage of corpuscles. Small portions were also used for the hemoglobin determinations.

Fifteen cubic centimeters of blood from each sample was used in the preparation of the protein-free filtrate which was prepared according to the method of Folin and Wu (61; 49, p. 237; 81, p. 367). The total proteins of the blood were removed by precipitation with tungstic acid (formed by the interaction of sodium tungstate and sulphuric acid) and filtration. A clear filtrate was obtained in every instance. Such a filtrate is suitable for the determination of non-protein nitrogen, urea, uric acid, creatinine, creatine, amino acids, chlorides and sugar.



## DETERMINATION OF THE BLOOD CONSTITUENTS

Total non-protein nitrogen was determined in a portion of each blood filtrate by a micro-Kjeldahl method, using a sulphuric acid and phosphoric acid mixture for the digestion, the nitrogen being determined colorimetrically after direct Nesslerization of the digestion mixture according to the Folin-Wu method (61; 49, p. 233; 81, p. 368).

Most of the urea nitrogen determinations were made by the Folin-Wu adaptation (61; 49, p. 237; 81, p. 370) of the Marshall method (110), in which the urea was decomposed to ammonium carbonate by the enzyme urease in the presence of phosphate. The ammonia was driven off by aeration and determined colorimetrically after Nesslerization. The urea was calculated by multiplying the urea nitrogen by the factor 2.15.

A few of the urea nitrogen determinations were made by Karr's method (100; 81, p. 373). The urea in the filtrate was converted by urease to ammonium carbonate, which was Nesslerized and compared colorimetrically with a standard urea solution similarly treated.

The uric acid determinations made on the blood obtained from pigs in Minnesota were performed according to the Folin-Wu adaptation (61) of the Folin-Denis method (54), in which the uric acid was isolated from the blood filtrate as the silver salt before being determined colorimetrically.

The uric acid determinations made on the blood obtained from pigs in Iowa were performed according to the Folin direct method (52) or Folin-Benedict method (49, p. 247; 81, p. 378), in which the phosphotungstic acid solution was reduced by the uric acid directly in the blood filtrate, giving a blue compound the uric acid of which was estimated colorimetrically.

The creatinine determinations were made by the Folin-Wu adaptations (61; 49, p. 243; 81, p. 375) of the Folin method (51), in which a portion of the blood filtrate was treated with alkaline picrate solution and the color which was developed compared with that of a standard in a colorimeter.

Creatine plus creatinine was determined by the method described by Folin and Wu (61; 49, p. 245; 81, p. 376), in which the creatine of the blood filtrate was transformed to creatinine by heating with dilute hydrochloric acid in an autoclave. The creatinine preformed and that from creatine was then determined by treating with alkaline picrate solution and the color developed compared with that of a standard in a colorimeter.

The sugar was determined by the Folin-Wu method (62; 81, p. 381), in which the protein-free blood filtrate was heated with alkaline copper solution, using a special "Folin-Wu sugar tube" to prevent reoxidation. The cuprous oxide formed was treated with a molybdate phosphate solution, a blue color being obtained, which was compared to that of a standard. Several modifications such as described by Folin (48) and Benedict (8, 9) were tried, but it was decided that the Folin-Wu method yielded the best results.

The serum from a portion of individual blood samples was separated from the corpuscles by centrifugalization and the serum obtained was used for the inorganic phosphorus and calcium determinations.

The inorganic phosphorus was determined by the Briggs modification (24) of the Bell-Doisy method (7), which depended upon the phosphate reacting with ammonium molybdate to form ammonium phosphomolybdate. The latter was reduced by hydroquinone in carbonate sulfite solution to a



blue compound which was compared in a colorimetric to a standard phosphate solution treated in the same manner.

The calcium was determined by the Clark-Collip modification (37) of the Kramer-Tisdall method (105), in which the calcium was precipitated directly from the serum as calcium oxalate. The calcium oxalate crystals were dissolved in normal sulphuric acid and titrated with one-hundredth normal potassium permanganate to a definite pink color, which should persist for at least one minute. A special micro-burette, with a scale interval of one-fiftieth of a cubic centimeter, was used for this titration.

In general, the determinations were made in duplicate and the colorimetric readings were checked against each other.

The blood filtrates were prepared as soon after the blood was drawn from the animal as they could be transported to the laboratory. Several days were required to make a complete analysis of each blood sample. During this period the blood filtrate was kept in an ice box. In some cases one or two drops of 10 per cent thymol in chloroform were added to the blood filtrate as a preservative.

The erythrocyte counts, volume percentage of corpuscles, hemoglobin determinations, leucocyte counts and differential counts are considered in Part II of this paper.

## RESULTS

### THE BLOOD OF NORMAL SWINE

Determinations were made of certain constituents of the blood of ninety-nine normal pigs that ranged in weight from 50 to 300 pounds. In addition, an analysis was made of the blood of a female pig two years old weighing 415 pounds.

The data resulting from these determinations are arranged in groups, in some cases according to the origin and in others according to the weight of the pigs.

In general, it can be said that the pigs from which these blood analyses were made were kept under the prevailing standard conditions of the farm. It is the common custom on farms to allow pigs to feed from self feeders, so that the animals consume as much food as their appetites dictate.

The results for the determinations of the blood constituents for the individual animals are recorded in tables in the original thesis, complete copies of which are in the Library of the University of Minnesota and the Library of Iowa State College.

Summary and treatment of the data for the concentration of the blood constituents secured from normal swine are recorded in tables 1 to 45, inclusive.

### *Minnesota Swine*

Group 1 consists of twenty-six normal pigs, four to five months of age, which were purchased from the stockyards at South Saint Paul in 1921. These pigs were brought to the Veterinary Division, University Farm, and immunized against hog cholera by the serum virus method, then kept for a twenty-one day quarantine period before blood samples were taken. During this period they were fed a ration consisting of corn, oats, red dog flour and tankage. The pigs were fed just enough

of these food stuffs to keep them at about a constant weight. The pigs averaged about 100 pounds in weight. The blood was obtained by tail bleeding.

Determinations of total non-protein nitrogen, creatinine and the creatine plus creatinine were made. The creatinine nitrogen and the percentage of the total non-protein nitrogen which the creatinine nitrogen represented was calculated.

Group 2 consists of four normal pigs that were purchased from the stock yards of South Saint Paul in August, 1928. They were brought to the University Farm and fed a ration which consisted of shelled corn and a protein mixture consisting of equal parts of tankage and linseed meal. The blood samples were obtained by tail bleeding.

The urea was calculated from the urea nitrogen, the uric acid nitrogen was calculated from the uric acid and the creatinine nitrogen was calculated from the creatinine. The percentages of the total non-protein nitrogen which the urea nitrogen, uric acid nitrogen and creatinine nitrogen represent are given.

Group 3 consists of four pigs which were slaughtered at the Midway abattoir in Saint Paul. The pigs averaged about 150 pounds in weight. No history was obtained as to the rations which these pigs had received. Determinations were made of non-protein nitrogen, urea nitrogen, uric acid, creatinine, creatine plus creatinine, sugar, inorganic phosphorus and calcium. Values for urea, uric acid nitrogen and creatinine nitrogen were calculated.

In May, 1929, three normal pigs were secured from the Animal Husbandry Department of the University Farm, Saint Paul, and taken to the Veterinary Division, where they were kept for a period of six weeks. During this time four blood samples were obtained from each pig and analyzed for non-protein nitrogen, uric acid nitrogen, creatinine, creatine plus creatinine, sugar, inorganic phosphorus and calcium. These pigs received a ration which consisted of shelled corn, a protein mixture of equal parts of tankage and linseed meal. Before being brought to the Veterinary Division they had access to rape pasture in addition to their regular ration. These pigs were between eight and nine months of age. The averages of these determinations are recorded under groups 4a, 4b and 4c. The urea, uric acid nitrogen and creatinine nitrogen are calculated. The percentages of the total non-protein nitrogen represented by the nitrogen of the various constituents are recorded.

Group 5 consists of five pigs which were slaughtered at a packing plant in South Saint Paul. These blood samples were obtained by throat bleeding at the time of slaughter. The averages of determinations of certain blood constituents are recorded.

Group 6 consists of five normal pigs which were purchased at the stock yards in South Saint Paul and brought to the Veterinary Division of the University Farm. These pigs averaged about 70 pounds in weight. The blood samples were obtained by tail bleeding.

### *Iowa Swine*

Group 7 consists of three normal pigs belonging to the Department of Veterinary Research, Iowa State College, Ames, Iowa. These pigs were fed a ration of shelled corn, middlings and shorts twice a day.

The amount of feed was regulated so that the animals made a slow constant gain in weight, but did not fatten. These pigs averaged about 90 pounds in weight.

Groups 8, 9, 10, 11 and 12 consist of forty-eight normal pigs which weighed from 180 to 300 pounds live weight. Group 12a consists of one female pig which weighed 415 pounds. These pigs were raised on different farms near Ames, Iowa, and were slaughtered at the College abattoir. The rations which these pigs received varied slightly, but in general consisted of corn, a supplemental protein mixture consisting of milk and tankage and a mineral mixture of 20 parts of limestone by weight, 20 parts of salt by weight, 37.5 parts of bone meal by weight, 2 pounds of iron oxide, 0.02 pound of potassium iodide, 0.03 pound of copper sulphate. This ration was fed in a self feeder. In addition they were allowed access to alfalfa pastures.

These animals were deprived of water and feed for 24 to 36 hours prior to slaughter. As a rule the stomach and intestine were devoid of feed and, in fact, very little fecal material was found in the lower intestinal tract. The bladder usually contained only small amounts of urine and frequently only a trace.

The groups have been arranged according to the weights of the animals. Group 8 consists of three pigs ranging in weight from 180 to 185 pounds. Group 9 consists of twenty-four pigs ranging in weight from 200 to 225 pounds. Group 10 consists of ten pigs ranging in weight from 228 to 249 pounds. Group 11 consists of six pigs ranging in weight from 252 to 272 pounds. Group 12 consists of five pigs ranging in weight from 280 to 300 pounds. Group 12a includes only one female pig which weighed 415 pounds.

The blood samples were obtained by throat bleeding at the time of slaughter.

#### THE BLOOD OF CHOLERA-INFECTED SWINE

Determinations were made of certain constituents of the blood of fifty-four cholera-infected pigs that ranged in weight from 40 to 90 pounds.

The data are grouped according to the origin of the pigs, as it was believed that the disease might vary in its virulence from different sources.

As a rule the pigs developed anorexia on the third or fourth day after inoculation with cholera virus so that when these animals were killed, usually on the seventh day, in some cases up to the eleventh day, they were on a starvation metabolism basis. The blood samples were obtained by throat bleeding when the animals were killed.

The data secured from cholera-infected swine for the concentration of certain blood constituents are recorded in groups 13 to 15b, inclusive.

In group 13 are recorded the results of determinations of blood constituents from a group of cholera-infected pigs which belonged to the Department of Veterinary Research, Iowa State College. These pigs averaged about 54 pounds in weight. Maximum, minimum and mean values are given for this group.

The data secured from the determination of blood constituents of twenty-seven cholera-infected pigs belonging to the Experiment Station of the United States Bureau of Animal Industry, located at Ames, Iowa, are recorded in group 14. These pigs averaged about three months and twelve days in age and 57 pounds in weight. They were raised in the vicinity of Ames. Maximum, minimum and mean values for this group are given.



In groups 15a and 15b are recorded the results of determinations of blood constituents from two groups of pigs, consisting of eight and nineteen animals, respectively, belonging to the plant of the Fort Dodge Serum Company at Fort Dodge, Iowa. These pigs originated from North Dakota, being purchased in that state by the Fort Dodge Serum Company and brought to their plant for the purpose of producing hog cholera virus. These pigs ranged in weight from 70 to 90 pounds. They were killed on the sixth day after being inoculated with four cubic centimeters of hog cholera virus. Maximum, minimum and mean values are recorded for each group.

## DISCUSSION

### NORMAL BLOOD

A considerable amount of the limited data available concerning the concentration of the various constituents of swine blood has been obtained by investigators in developing the technique of the various procedures for blood analyses. In most instances the mixed blood of animals has been used. Relatively small numbers of swine have been used for this purpose.

Hayden and Tubangui (83) determined the creatinine and sugar content in the blood of twenty normal pigs. They also studied the non-protein nitrogen, urea and uric acid in the blood of fourteen normal pigs.

Scheunert and Pelchrim (137) determined the sugar, rest nitrogen, urea nitrogen, creatinine and creatine plus creatinine in the blood of fifteen normal pigs.

Roderick and Schalk (132) made determinations of non-protein nitrogen on eight normal pigs, and urea nitrogen and sugar determinations on twelve normal pigs.

### *Total Non-protein Nitrogen*

The data recorded in groups 1 to 12 show a wide range in the values of non-protein nitrogen in the blood of the different groups of pigs and between members of the same group.

TABLE 1. *Maximum, minimum and mean values for total non-protein nitrogen in the blood of different groups of normal pigs*

Group	No. of animals	No. of determinations	Mgms. per 100 c.c. of blood		
			Maximum	Minimum	Mean
1	26	26	40.0	8.6	26.0
2	4	4	54.0	33.3	46.8
3	4	4	40.0	35.2	38.4
4a	1	4	37.0	20.6	31.5
4b	1	4	30.0	26.0	27.3
4c	1	4	35.2	22.2	28.6
5	5	5	40.0	18.0	31.7
6	4	4	22.0	13.5	17.3
7	4	4	40.0	20.0	31.0
8	3	3	37.5	30.8	34.5
9	23	23	58.0	20.0	31.3
10	10	10	43.0	19.0	29.4
11	6	6	40.4	21.8	29.5
12	5	5	48.0	24.0	35.0
12a	1	1	30.0	30.0	30.0



In table 1 are given the maximum, minimum and mean values for total non-protein nitrogen for the different groups.

There is shown in table 1 a considerable variation in the average values of total non-protein nitrogen between the various groups and also between the animals comprising the different groups. Repeated observations on the same animal showed wide variations, as may be noted in group 4a. The non-protein nitrogen in blood samples obtained from the same animal ranged between 20.6 and 37 milligrams per 100 c.c. of blood.

The average value for non-protein nitrogen for 107 blood samples obtained from 98 normal pigs was found to be 31.4 milligrams per 100 c.c., ranging from 8.6 to 58 milligrams.

In table 2 there is recorded a comparison of the results obtained by different investigators for total non-protein nitrogen determined from the blood of different animals, with the results which we obtained from the blood of normal swine.

TABLE 2. *Comparison of the average values for total non-protein nitrogen obtained by different investigators from the blood of various animals with our determinations on normal swine*

Investigator	Animal	Mgms. per 100 c.c. of blood		
		Maximum	Minimum	Mean
Haden and Orr (71) .....	dog			30.0
Morgulis and Edwards (114) .....	dog			34.8
Folin and Denis (58) .....	cat	67.0	31.0	52.6
Folin and Morris (60) .....	rat			38.0
Anderson, Honeywell, Santy and Pederson (4) .....	rat			45.2
Chanutin and Silvette (35) .....	rat	51.0	37.0	43.0
Folin and Denis (58) .....	rabbit			31.0
Watkins and Smith (153) .....	rabbit			20.3
Scheunert and Pelchrzim (137) .....	horse	30.0	21.4	29.0
Holt and Reynolds (89) .....	horse	42.53	16.5	25.82
Hayden and Fish (82) .....	horse			33.9
Folin and Denis (58) .....	horse			54.0
Scheunert and Pelchrzim (137) .....	cattle	39.4	26.3	28.74
Hayden and Fish (82) .....	cattle	35.0	25.0	
Anderson, Gayley and Pratt (3) .....	cattle	42.14	20.67	30.07
Scheunert and Pelchrzim (137) .....	sheep			31.0
Hayden and Tubangui (83) .....	swine	38.3	30.6	35.0
Scheunert and Pelchrzim (137) .....	swine	40.04	25.0	31.1
Folin and Denis (58) .....	swine			32.0
Roderick and Schalk (132) .....	swine	49.9	23.0	41.5
Our observations .....	swine	58.0	8.6	31.4

It may be seen from table 2 that the average of 31.4 milligrams of total non-protein nitrogen per 100 c.c. of blood is in close agreement with the results of other investigators. A wider range in the values of total non-protein nitrogen was observed in these investigations than was recorded by other investigators. (Folin and Denis (58)), (Hayden and Tubangui (83)), (Scheunert and Pelchrzim (137)).

The distribution of the values which we obtained for total non-protein nitrogen in the blood of normal swine is given in table 3.

TABLE 3. *Distribution of the values for total non-protein nitrogen in the blood of normal swine*

Mgms. per 100 c.c.	No. of determinations	Pctg. total no. determinations	Mgms. per 100 c.c.	No. of determinations	Pctg. total no. determinations
Below 15	4	3.7	35-40	15.0	14.0
15-20	5.0	4.7	40-45	10.0	9.3
20-25	24.0	22.4	45-50	2.0	1.9
25-30	20.0	18.7	50-55	3.0	2.9
30-35	23.0	21.5	Above 55	1.00	0.9

*Urea Nitrogen*

In table 4 are given the maximum, minimum and mean values for urea nitrogen, the percentage of total non-protein nitrogen which is represented by the urea nitrogen and the values for urea for the different groups of normal pigs.

TABLE 4. *Maximum, minimum and mean values for urea nitrogen, percentage of the total non-protein nitrogen, and urea in the blood of various groups of normal pigs*

Group number	No. of animals	Number of determinations	Urea N Mgms. per 100 c.c.			Pctg. total non-protein nitrogen			Urea Mgms. per 100 c.c.		
			Maximum	Minimum	Mean	Maximum	Minimum	Mean	Maximum	Minimum	Mean
2	4	4	19.8	12.4	16.7	60.0	25.0	37.8	42.7	26.7	36.0
3	4	4	17.6	16.0	16.6	46.0	39.0	42.0	38.0	34.0	35.8
4a	1	4	18.7	7.0	14.6	85.0	18.0	50.0	40.0	15.0	31.4
4b	1	4	20.3	10.6	16.5	68.0	40.0	59.0	43.8	23.0	35.7
4c	1	3	19.8	14.0	16.5	89.0	44.0	61.7	42.7	30.7	35.8
5	5	5	15.7	6.4	10.0	46.0	17.8	33.0	34.0	14.6	21.8
6	5	5	8.2	2.2	5.4	61.0	11.0	32.4	17.8	4.5	11.6
7	4	4	10.7	3.7	5.7	28.5	9.2	17.5	22.9	7.9	12.2
8	3	3	5.8	2.2	3.4	16.4	5.7	10.0	12.4	4.6	7.4
9	23	23	10.4	2.0	5.1	39.5	4.0	18.0	22.4	4.3	11.0
10	10	10	8.0	4.0	5.3	42.0	9.0	20.5	17.2	8.6	11.5
11	6	6	9.4	1.3	4.8	43.0	4.0	18.6	20.3	2.7	10.4
12	5	5	5.0	1.3	3.2	18.8	3.2	9.6	10.8	2.8	7.0
12a	1	1	3.0	3.0	3.0	10.0	10.0	10.0	6.5	6.5	6.5

The average value for urea nitrogen for 81 determinations on 73 normal pigs was 7.7 milligrams per 100 c.c. of blood, ranging from 1.3 to 20.3 milligrams. The average percentage which the urea nitrogen made up of the total non-protein nitrogen was 26.28, with variations between 3.2 and 89 per cent. The average value for urea calculated from the urea nitrogen was 16.9 milligrams per 100 c.c. of blood, with a minimum of 2.7 and a maximum of 48.0 milligrams.

In table 5 is recorded a comparison of the results obtained by different investigators for urea nitrogen from the blood of different animals, with the results which we obtained from the blood of normal swine.

TABLE 5. *Comparison of average values for urea nitrogen obtained by different investigators from the blood of different animals with our results on the blood of normal swine*

Investigator	Animal	Mgms. per 100 c.c. of blood		
		Maximum	Minimum	Mean
Haden and Orr (71).....	dog			11.7
Morgulis and Edward (114).....	dog	15.0	11.1	12.9
	New born			
Pucher (129).....	puppies			20.7
Folin and Denis (58).....	cat	37.0	20.0	30.3
Folin and Morris (60).....	rat			10.25
Anderson, Honeywell, Santy and				
Pederson (4).....	rat			15.6
Folin and Denis (58).....	rabbit			13.0
	rabbit			
	pregnant			
Watkins and Smith (153).....	doe			11.0
	lactating			
Watkins and Smith (153).....	doe			18.0
Scheunert and Pelchrzim (137).....	horse	15.0	7.5	9.64
Holt and Reynolds (89).....	horse	25.0	7.5	14.81
Hayden and Fish (82).....	horse			18.7
Folin and Denis (58).....	horse			28.0
Brocq-Rousseau, Roussel and Gallot				
(26).....	horse	45.3	16.5	31.4
Scheunert and Pelchrzim (137).....	cattle	23.7	10.5	18.09
Hayden and Fish (82).....	cattle	27.0	5.3	12.21
Anderson, Gayley and Pratt (3).....	cattle	21.64	4.4	12.94
Folin and Denis (58).....	cattle	14.0		
Scheunert and Pelchrzim (137).....	sheep	23.1	7.5	11.8
Folin and Denis (58).....	sheep			13.0
Hayden and Tabangui (83).....	swine	27.3	15.35	19.6
Scheunert and Pelchrzim (137).....	swine			15.35
Folin and Denis (58).....	swine			14.0
Roderick and Schalk (132).....	swine	23.4	11.0	17.3
Our observations.....	swine	20.3	1.3	7.7

The distribution of our values for urea nitrogen in the blood of normal swine is recorded in table 6.

TABLE 6. *Distribution of the values for urea nitrogen in the blood of normal swine*

Mgms. per 100 c.c.	No. of de- termina- tions	Percentage total no. determina- tions	Mgms. per 100 c.c.	No. of de- termina- tions	Percentage total no. determina- tions
Below 5	32.0	40.0	15-20	16.0	1.0
5-10	27.0	33.0	Above 20	19.5	1.2
10-15	6.0	7.3			

The mean value for urea nitrogen is lower than that recorded by other investigators for normal animals. In several of the groups of pigs comprising more than 20 per cent of the total number of our determinations, the values for urea nitrogen are between 14 and 20 milligrams per 100 c.c. of blood, in which range are found the average values of other investigators. The maximum value of 20.3 milligrams is lower than the maximum values recorded by Hayden and Tubangui (83) and Roderick and Schalk (132), which were 27.3 and 23.4 milligrams, respectively.

The accuracy of our technique for the determination of urea nitrogen was investigated. Two urea solutions, one (solution A) containing one-tenth of a milligram of urea per 10 c.c. and another (solution B) containing one milligram of urea per 10 c.c., were prepared. Determinations of the urea nitrogen in 10 c.c. of each solution were made.

The results for solution A gave a value of 0.0438 milligrams of urea nitrogen or 0.094 milligram of urea, which was 94 per cent of the amount present. The results for solution B gave a urea nitrogen value of 0.4545 milligram, equivalent to 0.98 milligram of urea, which was 98 per cent of the amount present.

Folin and Berglund (53) obtained data on the amino-acid absorption and urea accumulation which in their opinion indicated that the urea formation is a function of all mammalian tissues, including the blood corpuscles, and is not predominantly localized in the liver.

Jansen (96) by perfusing the liver, showed that the liver does play an important part in the formation of urea from amino-acids.

Bollman, Mann, and Magath (23) studied the deamination of the blood, urine and tissues of a number of dogs surviving removal of the liver from 8 to 30 hours. In no case could the occurrence of deamination be demonstrated after the liver had been removed. It was demonstrated that no urea was formed in the absence of the liver. If amino-acids were injected into these animals the entire amount of amino-acid nitrogen was recovered unchanged in the blood. They concluded that deamination of amino-acids in the body of the dog was entirely dependent on the presence of the liver, since deamination ceased completely as soon as the liver was removed.

Johnson (98) found that the average number of lobules in the pig's liver was 702,000. He quotes Mall as having obtained 480,000 as the average number of lobules in the liver of the dog.

A greater number of liver lobules indicates a greater amount of interlobular connective tissue. It is well known that the liver of the pig contains relatively more connective tissue than does the liver of other animals. Because of this greater amount of interlobular connective tissue the lobules of the liver in the pig are easily discernable macroscopically.

Johnson (98) states that the size of the lobule of an adult pig's liver is very variable, great differences existing in any individual liver. The smallest lobules may be no larger than 0.5 millimeter in diameter; the largest ones may be 2 millimeters or over. Assuming that the shapes of the large and small lobules are approximately similar, it is evident that the largest lobules must be as much as sixty-four times greater by volume than the smallest ones. The average volume of the liver lobule is dependent to a certain degree upon the size of the liver, thus in small livers the average volume is less than in larger ones. The total number of lobules in the pig's



liver is also quite variable. This can be readily observed when examining isolated lobules of different livers of approximately the same weight.

Could it be that, correlated with the relatively large amount of connective tissue in a pig's liver and the variations in size and volume of the lobules, there is an instability of physiologic activity?

Hubbard (93) studied the urea and creatinine concentration in the blood and found that urea and creatinine concentrations paralleled each other. He stated that there was not infrequent evidence of rather sudden changes in urea concentration probably associated with variations in the rate of urea formation. In the table showing his results he cites cases having urea nitrogen values between 0.0 and 4.9 milligrams, and between 0.0 and 8 milligrams, with creatinine ranging between 1.33 and 1.44 milligrams per 100 c.c. of blood.

### *Uric Acid*

In table 7 are given the maximum, minimum and mean values for uric acid, uric acid nitrogen, and the percentage of total non-protein nitrogen represented by the uric acid nitrogen, in the blood of various groups of pigs.

TABLE 7. *Maximum, minimum and mean values for uric acid, and the uric acid nitrogen in milligrams and percentage of total non-protein nitrogen in the blood of various groups of normal swine*

Group number	Number of animals	Number of deter- minations	Mgms. per 100 c.c. of blood						Percentage of total non-protein nitrogen		
			Uric acid			Uric acid nitrogen					
			Maximum	Minimum	Mean	Maximum	Minimum	Mean	Maximum	Minimum	Mean
2	4	4	1.7	1.2	1.44	0.5	0.4	0.43	1.2	0.76	0.98
3	4	4	1.08	1.04	1.05	0.35	0.34	0.34	1.0	0.86	0.9
4a	1	3	1.3	0.35	0.98	0.45	0.11	0.33	1.3	0.32	0.92
4b	1	3	1.31	0.62	1.02	0.43	0.2	0.33	1.4	0.7	1.17
4c	1	3	1.2	0.72	0.95	0.4	0.23	0.31	1.7	0.76	1.15
5	5	5	0.76	0.65	0.7	0.25	0.21	0.22	1.0	0.5	0.7
6	3	3	1.2	1.15	1.18	0.41	0.38	0.39	3.0	2.0	2.6
7	4	4	2.2	1.0	1.4	0.73	0.33	0.45	1.8	0.8	1.4
8	3	3	7.14	3.07	4.8	2.3	1.0	1.6	6.7	2.7	4.7
9	23	23	4.06	0.58	2.3	1.3	0.2	0.77	5.8	0.6	2.78
10	10	10	4.2	0.46	3.4	1.4	0.15	0.8	7.2	0.35	3.0
11	6	6	3.2	0.32	1.4	1.0	0.18	0.45	3.0	0.4	1.5
12	5	5	3.5	0.32	2.3	1.2	0.1	0.75	3.0	0.44	2.1
12a	1	1	3.3	3.3	3.3	1.0	1.0	1.0	3.6	3.6	3.6

The average value for uric acid from 78 determinations on 71 normal pigs was 1.95 milligrams per 100 c.c. of blood, ranging from 0.32 to 7.14 milligrams. The average for uric acid nitrogen was 0.58 milligram per 100 c.c. of blood and varied between 0.1 and 2.3 milligrams. The uric acid

nitrogen averaged 2.12 per cent of the total non-protein nitrogen, ranging from 0.32 to 7.2 per cent.

A comparison of the data obtained by different investigators for uric acid in the blood of different animals with our results on normal swine is shown in table 8.

TABLE 8. *Comparison of average values for uric acid obtained from the blood of different animals with our results on the blood of normal swine*

Investigator	Animal	Mgms. per 100 c.c. of blood		
		Maximum	Minimum	Mean
Haden and Orr (71).....	dog			1.5
Benedict .....	dog			0.46
Folin and Denis (58).....	cat			0.2
Folin and Morris (60).....	rat			2.0
Anderson, Honeywell, Santy and Pederson (4) .....	rat			1.86
Folin and Denis (58).....	rabbit			0.05
Holt and Reynolds (89).....	horse	3.0	0.9	1.4
Hayden and Fish (82).....	horse			0.69
Folin and Denis (58).....	horse			0.05
Hayden and Fish (82)—(Folin's direct method) .....	cow			2.04
Hayden and Fish (82)—(Isolation method of Folin) .....	cow			0.06
Anderson, Gayley and Pratt (3)—(Benedict's method) .....	cow			2.08
Folin and Denis (58).....	cow			0.2
	defibrinated ox			
Benedict (13) .....	blood	0.67	0.41	0.5
Folin and Denis (58).....	sheep			0.05
Hayden and Tubangui (83).....	swine	2.5	1.73	2.06
Folin and Denis (58).....	swine			0.05
Our results .....	swine	7.14	0.32	1.95

The average which was obtained in this study for the concentration of uric acid in the blood of swine agrees quite closely with the average obtained by Hayden and Tubangui (83). Again our data show a wider variation than is recorded by other investigators.

Harding and collaborators (78) made the observation that diets high in fat producing a ketosis invariably raised the uric acid content of the blood, and that often the rise in uric acid was accompanied by an increase in the non-protein nitrogen which was not attributable to an augmentation of the blood urea.

It must be borne in mind that the high uric acid values and the low urea nitrogen values in our studies are restricted for the most part to groups 8, 9, 10, 11 and 12. The animals comprising these groups were slaughtered at the college abattoir. Feed and water had been withheld from these animals for 24 to 36 hours previous to slaughter. In addition to having feed and water withheld, they had been loaded on a truck and hauled to the abattoir.

Bulmer, Eagles, and Hunter (31), Benedict, Newton, and Bahre (16) have reported the isolation of a new substance (thiasine) from blood, which gives a blue color with uric acid reagents. This compound is supposed to be responsible for the occasional excess estimation of uric acid by the direct method as compared with results by the indirect method. It is possible that Folin's improved phosphotungstic reagent (49, p. 247) may not react to this substance.

Holbrook and Haskins (88), in studying the blood uric acid in nephritis, presented data to show urea nitrogen retention in the blood with normal uric acid values. They considered urea nitrogen of 16 to 24 milligrams as the range of early retention. They concluded that uric acid was not retained in the blood at an earlier stage of nephritis than was urea. They considered that creatinine estimations were much more valuable and agreed approximately with urea results and served as a check upon the latter. They concluded that urea estimations were the most reliable and significant of the blood chemistry findings in nephritis.

Myers, Fine, and Lough (120), on the other hand, presented a series of 30 cases with high values for the uric acid content of the blood, but without a corresponding retention of urea and creatinine. They concluded that uric acid was of considerable value as an early diagnostic test for "early interstitial nephritis."

The distribution of the values which we obtained for uric acid in the blood of normal swine is recorded in table 9.

TABLE 9. *Distribution of the values for uric acid in the blood of normal swine*

Mgms. per 100 c.c.	No. of determinations	Percentage total no. determinations	Mgms. per 100 c.c.	No. of determinations	Percentage total no. determinations
Below 0.5	4	5.1	2.5-3.0	4	5.1
0.5-1.0	13	16.7	3.0-3.5	9	11.5
1.0-1.5	27	34.7	3.5-4.0	8	10.3
1.5-2.0	5	6.4	4.0-4.5	3	3.8
2.0-2.5	4	5.1	Above 4.5	1	1.3

Slightly more than 51 per cent of the determinations for uric acid gave values between 0.5 and 1.5 milligrams per 100 c.c. of blood.

Bollman, Mann, and Magath (22) found large amounts of uric acid in the urine of dogs following hepatectomy, which also gave rise to an increase in the uric acid content of the blood. The uric acid excreted by the hepatectomized animal was of the same magnitude as the allantoin excreted by the normal dog. The lack of destruction of uric acid following removal of the liver indicated that this organ was responsible for its destruction in the normal animal.

### *Preformed Creatinine*

In table 10 are recorded data for creatinine and creatinine nitrogen in milligrams and percentage of total non-protein nitrogen in the blood of different groups of normal pigs.

TABLE 10. *Maximum, minimum and mean values for creatinine, creatinine nitrogen, and percentage of total non-protein nitrogen represented by the creatinine nitrogen in the blood of various groups of normal pigs*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. of blood						Creatinine nitrogen percentage total non-protein nitrogen		
			Creatinine			Creatinine N			Maximum	Minimum	Mean
			Maximum	Minimum	Mean	Maximum	Minimum	Mean			
1	26	26	1.6	1.0	1.3	0.6	0.4	0.48	5.0	1.0	2.2
2	4	4	1.3	1.2	1.2	0.5	0.5	0.5	1.7	0.9	1.2
3	4	4	1.5	1.5	1.5	0.6	0.57	0.58	1.6	1.4	1.4
4a	1	4	2.0	1.2	1.4	0.7	0.45	0.52	2.1	1.3	1.7
4b	1	4	1.6	1.3	1.4	0.57	0.5	0.55	2.1	1.6	1.9
4c	1	4	1.6	1.2	1.4	0.6	0.5	0.55	2.0	1.6	1.9
5	5	5	2.0	1.5	1.8	0.75	0.57	0.67	3.0	1.4	2.2
6	5	5	1.5	1.4	1.4	0.57	0.53	0.55	4.2	2.0	3.3
7	4	4	1.75	1.4	1.5	0.66	0.53	0.58	2.4	1.5	1.8
8	3	3	2.0	1.4	1.6	0.76	0.5	0.61	2.2	1.4	1.8
9	21	21	2.6	1.2	1.8	1.0	0.45	0.68	3.3	1.1	2.3
10	10	10	2.1	1.2	1.8	0.8	0.46	0.7	3.6	1.2	2.4
11	6	6	2.7	1.2	2.0	1.0	0.44	0.75	3.5	1.3	2.7
12	5	5	2.0	1.6	1.8	0.76	0.63	0.70	3.1	1.3	2.1
12a	1	1	2.0	2.0	2.0	0.76	0.76	0.76	2.5	2.5	2.5

The average value for creatinine from 106 determinations on 97 normal pigs was 1.6 milligrams per 100 c.c. of blood, ranging from 1 to 2.7 milligrams. For creatinine nitrogen the average was 0.66 milligrams per 100 c.c. of blood, ranging between 0.44 and 1 milligram. The creatinine nitrogen averaged 2.2 per cent of the total non-protein nitrogen and varied from 0.9 to 5.1 per cent.

Folin (50, p. 274) stated that the creatinine content of the blood is normally remarkably constant, as might be expected in view of the fact that the endogenous production of creatinine is by far the largest source of urinary creatinine.

Our data show creatinine in the blood of swine, to be the most constant of the constituents which we have determined.

Myers and his associates (36) emphasized the point that the amount of increase of the blood creatinine should be a safer index to the decrease in the permeability of the kidney than the urea, because urea formation was largely exogenous and subject to greater fluctuations.

A comparison of the average values for the creatinine in the blood of various species as determined by different investigators, is made with our results on the blood of swine, in table 11.

The results of our determinations of creatinine are in fair agreement with the results obtained by other investigators for the blood of swine. The figures also show that the concentration of creatinine in swine blood is quite similar to that of other animals.



TABLE 11. *Comparison of average values for creatinine obtained from the blood of different species of animals by various investigators with our results of the blood of normal swine*

Investigator	Animal	Mgms. per 100 c.c. of blood		
		Maximum	Minimum	Mean
Haden and Orr (74).....	dog			1.5
Morgulis and Edwards (114).....	dog	1.7	1.5	1.5
Folin and Denis (57).....	cat			1.2
Anderson, Honeywell, Santy and Pederson (4).....	rat			1.27
Chanutin and Silvette (35).....	rat	2.1	1.1	1.4
Folin and Denis (57).....	rabbit			1.0
Scheunert and Pelchrzim (137).....	horse	1.7	1.2	1.27
Holt and Reynolds (89).....	horse	3.0	0.9	1.4
Hayden and Fish (82).....	horse			1.81
Scheunert and Pelchrzim (137).....	cattle	1.8	1.4	1.61
Hayden and Fish (87).....	cattle	2.0	1.0	
Anderson, Gayley and Pratt (3).....	cattle	1.94	1.19	1.42
	beef			
Folin and Denis (57).....	blood			2.0
Greenwald and McGuire (68)	beef			
(Folin's method).....	blood			2.57
Greenwald and McGuire (68) (using	beef			
their own method).....	blood			1.61
Scheunert and Pelchrzim (137).....	sheep	1.3	1.2	1.26
	mixed			
	blood of			
Folin and Denis (57).....	sheep			1.2
Hayden and Tubangui (83).....	swine	2.0	1.14	1.42
Scheunert and Pelchrzim (137).....	swine	1.94	1.62	1.78
	mixed			
	blood of			
Folin and Denis (57).....	swine			1.3
Our observations.....	swine	2.7	1.0	1.6

The distribution of the values for creatinine which we obtained is shown in table 12.

TABLE 12. *Distribution of the values for creatinine in the blood of normal swine*

Mgms. per 100 c.c.	No. of determinations	Percentage total no. determinations	Mgms. per 100 c.c.	No. of determinations	Percentage total no. determinations
1.0-1.5	45	42.5	2.0-2.5	21	19.8
1.5-2.0	38	35.9	Above 2.5	2	1.8

More than 78 per cent of the creatinine values lie between 1 and 2 milligrams per 100 c.c. of blood.

*Creatine Plus Creatinine*

The data collected for the concentration of creatine plus creatinine in the blood of different groups of normal pigs are given in table 13.

TABLE 13. *Maximum, minimum and mean values for creatine plus creatinine in the blood of various groups of normal swine*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. of blood		
			Maximum	Minimum	Mean
1	25	25	6.0	2.3	3.7
2	4	4	5.0	4.2	4.7
3	4	4	7.5	4.0	5.07
4a	1	4	5.0	2.8	4.3
4b	1	3	4.8	4.0	4.3
4c	1	4	4.8	3.0	4.2
5	5	5	4.8	3.1	4.0
6	4	4	7.7	2.6	4.0
7	4	4	5.8	4.2	4.8
8	3	3	6.3	5.3	5.9
9	21	21	12.0	4.4	6.1
10	10	10	7.0	4.4	6.0
11	5	5	6.6	5.2	5.9
12	5	5	8.1	4.8	6.5
12a	1	1	7.7	7.7	7.7

The average value for creatine plus creatinine for 102 determinations on 94 normal pigs was 5 milligrams per 100 c.c. of blood, ranging from 2.3 to 12 milligrams.

A comparison of the results obtained by different investigators for the creatine plus creatinine concentration in the blood of various species, with our results for the blood of swine, is given in table 14.

TABLE 14. *Comparison of creatine plus creatinine values in the blood of different species obtained by different investigators with values we obtained for swine*

Investigator	Animal	Mgms. per 100 c.c. of blood		
		Maximum	Minimum	Mean
Folin and Denis (57).....	cat			8.0
Anderson, Honeywell, Santy and Pederson (4).....	rat			6.42
Chanutin and Silvette (35).....	rat	6.7	5.1	5.7
Folin and Denis (57).....	rabbit			10.0
Scheunert and Pelchrim (137).....	horse	6.6	5.0	5.9
Scheunert and Pelchrim (137).....	cattle	10.4	5.7	7.71
Folin and Denis (57).....	beef			
	blood			11.0
Scheunert and Pelchrim (137).....	sheep	5.3	3.8	4.14
Folin and Denis (57).....	sheep			9.0
Scheunert and Pelchrim (137).....	swine	8.7	5.16	6.32
Folin and Denis (57).....	swine			9.0
Our determinations .....	swine	12.0	2.3	5.0

The average value obtained by us for creatine plus creatinine is somewhat lower than the average reported by Scheunert and Pelchrim (137) and Folin and Denis (57) in the blood of swine.

The distribution of the values for creatine plus creatinine which we obtained is shown in table 15.

TABLE 15. *Distribution of the values for creatine plus creatinine in the blood of normal swine*

Mgms. per 100 c.c.	No. of deter- mina- tions	Percentage total no. determina- tions	Mgms. per 100 c.c.	No. of deter- mina- tions	Percentage total no. determina- tions
Below 3	7	6.86	6-7	16	15.7
3-4	13	12.76	7-8	5	4.9
4-5	31	30.4	8-9	3	2.9
5-6	26	25.5	Above 9	1	0.98

Approximately 56 per cent of the determinations gave values for creatine plus creatinine between 4 and 6 milligrams per 100 c.c. of blood.

Folin (50, p. 274) states that the figures for blood creatine must necessarily be more uncertain than for blood creatinine, because of the heat which must be applied to the blood filtrate for the transformation of the creatine into creatinine. He concluded that creatine determinations were not of much clinical value. The creatinine determinations are of considerable clinical importance.

### Sugar

The data which we have accumulated for the concentration of sugar in the blood of various groups of normal pigs are recorded in table 16.

TABLE 16. *Maximum, minimum and mean values for blood sugar in various groups of normal swine*

Group Number	Number of animals	Number of determina- tions	Mgms. per 100 c.c. of blood		
			Maximum	Minimum	Mean
2	2	2	90	76	83.0
3	4	4	90	80	86.0
4a	1	3	220	62	119.0
4b	1	3	65	46	56.3
4c	1	4	105	38	80.0
6	5	5	85	41	68.0
7	4	4	222	200	210.0
8	3	3	103	52	75.0
9	22	22	420	55	132.0
10	10	10	500	63	186.0
11	6	6	347	38	177.0
12	5	5	66	55	61.0
12a	1	1	62	62	62.0

The average value for the blood sugar level from 72 determinations on 65 normal pigs was 128 milligrams per 100 c.c. of blood. The values ranged from 38 to 500 milligrams.

The maximum recorded in our results is higher than that recorded by other investigators for any species. Holt and Reynolds (89) reported sugar values for the blood of normal horses to range between 78 and 370 milligrams per 100 c.c., with an average of 109 milligrams. Scheunert and Pelchrzim (137) found the blood sugar in horses to range between 76.9 and 200 milligrams per 100 c.c., with an average of 138.5 milligrams.

Ruggeri (135) found that the blood sugar in castrated rabbits ranged from 280 to 320 milligrams per 100 c.c. following the injection of diuretics.

Hewitt (86) found the blood sugar level in cattle to vary between 52 and 362 milligrams per 100 c.c.

A comparison of the results obtained by different investigators for the concentration of sugar in the blood of various species with our results for the blood of swine is given in table 17.

TABLE 17. *Comparison of sugar values in the blood of different species obtained by different investigators with results of our determination in the blood of normal swine*

Investigator	Animal	Mgms. per 100 c.c. of blood		
		Maximum	Minimum	Mean
Haden and Orr (71).....	dog			76.0
Haden and Orr (200 analyses) (74)....	dog			82.0
Morgulis and Edwards (114).....	dog	106.3	74.0	96.7
Voegtlin, Dunn and Thompson (147)..	rat			113.0
Anderson, Honeywell, Santy and Pederson (4).....	rat			122.0
Chanutin and Silvette (35).....	rat	154.0	115.0	136.0
	rabbit (normal doe)			124.0
Watkins and Smith (153).....	rabbit (pregnant doe)			110.0
Watkins and Smith (153).....	rabbit (lactating doe)			113.0
Watkins and Smith (153).....	castrated rabbit after injection of diuretics)	320.0	280.0	
Ruggeri (135) .....	horse	200.0	76.9	138.5
Scheunert and Pelchrzim (137).....	horse	370.0	78.0	109.0
Holt and Reynolds (89).....	horse			102.9
Hayden and Fish (82).....	horse	120.0	62.0	
Schwarz (138) .....	cattle	100.0	62.5	76.65
Scheunert and Pelchrzim (137).....	cattle	70.0	30.0	46.52
Hayden and Fish (82).....	cattle	142.0	43.2	64.1
Anderson, Gayley and Pratt (3).....	cattle	362.0	52.0	95.9
Hewitt (86) .....	cattle	177.0	44.0	82.0
Schwarz (138) .....	sheep	88.9	50.9	65.6
Scheunert and Pelchrzim (137).....	swine	109.0	76.0	90.1
Hayden and Tubangui (83).....	swine	106.7	80.0	92.8
Scheunert and Pelchrzim (137).....	swine	500.0	38.0	128.0
Our determinations .....	swine			



The distribution of the values for blood sugar which we found is given in table 18.

TABLE 18. *Distribution of values for blood sugar in normal swine*

Mgms. per 100 c.c.	No. of de- termina- tions	Percentage total no. determina- tions	Mgms. per 100 c.c.	No. of de- termina- tions	Percentage total no. determina- tions
Below 40	2	2.8	100-110	5	7.0
40-50	2	2.8	110-125	1	1.4
50-60	5	7.0	200-225	8	11.1
60-70	12	16.6	250-300	1	1.4
70-80	14	19.4	300-350	2	2.8
80-90	10	13.9	400-500	6	8.3
90-100	4	5.5			

Approximately 50 per cent of the determinations for sugar range between 60 and 90 milligrams per 100 c.c. of blood.

Tatum (143) presented data which showed that hemorrhage produced, if its grade was properly chosen, a reciprocal change in both a fall in the alkaline reserve capacity of whole blood and rise in blood and plasma sugar concentration. One effective cause of sugar changes in the blood appeared to be a disturbance in the acid-base balance in tissue cells indicated by that of the circulating blood in general. Hemorrhage produced hyperglycemia more readily when the total bodily alkaline reserve was diminished by administration of acid, and less readily when proper amounts of alkali were given.

Biester (19) reported the case of a pig which showed the presence of glycosuria as high as 6.6 per cent, which he concluded might have been diabetes induced by necrotic enteritis. Autopsy showed degeneration of the islands of Langerhan in the pancreas, and hepatic lesions.

### *Inorganic Phosphorus*

We are concerning ourselves only with the determination of the inorganic phosphorus of the serum, which, according to Howland and Kramer (92), represents the orthophosphate content of the serum, the only form of phosphate which can react with calcium to form tertiary calcium phosphate. Howland and Kramer conclude that the inorganic phosphorus represents a definite chemical entity since it is present in nearly constant amounts in the same individual as well as in normal individuals of the same age.

The data which we have obtained as the result of our determinations of the concentration of inorganic phosphorus in the blood of various groups of pigs are recorded in table 19.

As a result of 69 analyses on 61 normal pigs we obtained an average of 7.26 milligrams per 100 c.c. of serum, with a minimum of 4.1 and a maximum of 11.7 milligrams.

TABLE 19. *Maximum, minimum and mean values for inorganic phosphorus in the blood of various groups of normal swine*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. serum		
			Maximum	Minimum	Mean
3	4	4	6.6	4.1	5.8
4a	1	4	7.0	6.0	6.3
4b	1	3	6.6	5.8	6.2
4c	1	4	5.8	5.2	5.5
5	4	4	8.6	4.8	7.4
7	3	3	8.9	8.5	8.7
8	3	3	8.3	6.1	6.9
9	23	23	11.0	5.0	7.5
10	10	10	11.7	5.6	8.0
11	5	5	10.0	5.8	7.9
12	5	5	10.0	5.6	6.8
12a	1	1	5.0	5.0	5.0

A comparison of the results obtained by different investigators for the concentration of inorganic phosphorus in the serum of various species with our results for the serum of swine is given in table 20.

TABLE 20. *Comparison of inorganic phosphorus values in the blood of different species obtained by different authors with the results of our determinations in the blood of normal swine*

Investigator	Animal	Mgms. per 100 c.c. of serum		
		Maximum	Minimum	Mean
Abderhalden (1).....	dog			3.5
Abderhalden (1).....	cat			3.1
Brown (28).....	rabbit			4.51
Harnes (79).....	rabbit	6.82	4.96	
Abderhalden (1).....	horse			3.3
Anderson, Gayley and Pratt (3).....	cattle	6.17	3.09	4.46
Robinson and Huffman (131).....	cattle	8.99	3.0	5.87
	cattle (timothy hay)			
Hart et al (80).....	cattle (alfalfa hay)			2.57
Hart et al (80).....	hay			
Meigs, Blatherwick and Cary (112)....	cattle	8.8	3.8	4.62
Abderhalden (1).....	cattle			5.3
Abderhalden (1).....	sheep			2.7
Abderhalden (1).....	swine			3.2
Our determinations .....	swine	11.7	4.1	2.3
				7.26

The average value for inorganic phosphorus of 7.26 milligrams per 100 c.c. of serum is higher than that recorded by other investigators for domestic animals. The maximum value of 11.7 is 2.71 milligrams higher than the maximum of 8.99 obtained by Robinson and Huffman (131) for cattle.

The distribution of the values for inorganic phosphorus which we obtained for swine blood is given in table 21.

TABLE 21. *Distribution of inorganic phosphorus values for swine blood*

Mgms. per 100 c.c. of serum	Number of deter- mina- tions	Percent- age total no. deter- mina- tions	Mgms. per 100 c.c. of serum	Number of deter- mina- tions	Percent- age total no. deter- mina- tions
Below 4.5	1	1.5	7.5-8.0	2	2.9
4.5-5.0	1	1.5	8.0-8.5	4	5.9
5.0-5.5	5	7.35	8.5-9.0	7	10.3
5.5-6.0	10	14.7	9.0-9.5	2	2.9
6.0-6.5	12	17.63	9.5-10.0	3	4.41
6.5-7.0	8	11.78	10.0-11.0	6	8.82
7.0-7.5	4	5.9	Above 11.0	3	4.41

Approximately 50 per cent of the determinations are between 5 and 7 milligrams per 100 c.c. of serum.

Palmer, Cunningham, and Eckles (124), in studying the normal variations in the inorganic phosphorus of dairy cattle, found that wide fluctuations may occur from day to day in consecutive three-day tests even when the samples were taken under presumably identical physiological conditions. Of the three physiological factors studied, namely, exercise, food ingestion and water drinking, only the first gave results of sufficient significance to account for the abnormally great fluctuations. Their studies showed that vigorous exercise usually caused an increase in the inorganic phosphorus, followed within a half-hour by a marked decrease to a point below the "before exercise" level. They concluded that the increase in inorganic blood phosphorus as a result of exercise was possibly caused by a breakdown in hexose phosphates, to supply energy, thereby releasing inorganic phosphorus into the blood. The decrease which followed would by analogy, be the result of a resynthesizing of hexose phosphates. It would seem that any severe resistance of an animal to the act of bleeding would cause changes in the inorganic phosphorus of the blood.

### *Calcium*

The literature on the variations which occur in the concentration of calcium in the blood under normal conditions is not large. Meigs, Blatherwick and Cary (112) concluded that their experience supported the observations of Kramer and Tisdall (104) in that the calcium of mammalian blood was contained in the plasma, and to a negligibly small extent, if at all, in the corpuscles.

Our results for the concentration of calcium in the serum of various groups of swine are recorded in table 22.

The average value for the calcium concentration in the serum of normal pigs which we obtained was 12.39 milligrams per 100 c.c. for 71 determinations on 63 pigs. The values ranged between a minimum of 6.5 and a maximum of 20 milligrams.

TABLE 22. *Maximum, minimum and mean values for the concentration of calcium in the blood of various groups of normal swine*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. of serum		
			Maximum	Minimum	Mean
3	4	4	19.0	17.0	17.6
4a	1	4	17.0	6.5	12.5
4b	1	3	17.5	14.0	15.5
4c	1	4	18.0	14.0	16.5
5	4	4	13.0	7.0	10.0
7	4	4	14.0	10.0	12.4
8	3	3	14.3	9.8	12.0
9	23	23	14.0	9.0	11.1
10	10	10	16.0	8.0	11.0
11	6	6	20.0	10.0	13.5
12	5	5	19.0	7.2	12.4
12a	1	1	10.4	10.4	10.4

The average of 12.39 milligrams per 100 c.c. of serum is 0.24 milligrams lower than the average of 12.63 obtained by Anderson, Gayley and

TABLE 23. *Calcium values obtained by different investigators for various species compared with our results for normal swine*

Investigator	Animal	Mgms. per 100 c.c. of serum		
		Maximum	Minimum	Mean
Abderhalden (1).....	dog			8.1
Abderhalden (1).....	cat			7.8
Teich (146).....	guinea pig	13.0	6.0	7.3
Culhane (40).....	rabbit	17.12	14.31	15.47
Brown (28).....	rabbit			15.6
Harnes (79).....	rabbit	18.5	14.5	
Abderhalden (1).....	rabbit			8.3
Clark (38).....	horse	12.3	9.0	11.26
Rona and Takahashi (134).....	horse			12.0
Abderhalden (1).....	horse			7.9
Anderson, Gayley and Pratt (3).....	cattle	16.18	9.96	12.63
Robinson and Huffman (131).....	cattle	14.17	7.7	11.0
	cattle (timothy hay)	25.0	20.0	
Hart et al (80).....	cattle (alfalfa hay)			10.0
	pregnant cow non-lactating			9.6
Meigs, Blatherwick and Cary (112)....	lactating cow not pregnant			9.8
Meigs, Blatherwick and Cary (112)....	cattle			7.9
Abderhalden (1).....	sheep			8.4
Abderhalden (1).....	swine			8.7
Our determinations .....	swine	20.0	6.5	12.39



Pratt (3) for cattle. Hart et al (80) found serum calcium values of 20 to 25 milligrams per 100 c.c. in cattle fed on a ration of timothy hay.

A comparison of our results for calcium values in the blood of swine with those of various investigators for different animals is recorded in table 23.

The distribution of the values for serum calcium obtained from our observations on normal swine is given in table 24.

TABLE 24. *Distribution of values for serum calcium in normal swine*

Mgms. per 100 c.c. of serum	Number of deter- mina- tions	Percent- age total no. deter- mina- tions	Mgms. per 100 c.c. of serum	Number of deter- mina- tions	Percent- age total no. deter- mina- tions
Below 7	1	1.4	13-14	6	8.5
7-8	2	2.8	14-15	10	14.0
8-9	4	5.62	15-16	4	5.62
9-10	12	17.0	16-17	3	4.22
10-11	7	9.85	17-18	4	5.62
11-12	6	8.5	18-19	2	2.8
12-13	7	9.85	Above 19	3	4.22

Forty-five per cent of the determinations averaged between 9 and 13 milligrams of calcium per 100 c.c. of serum.

#### CHOLERA-INFECTED BLOOD

With the exception of the work reported by Roderick and Schalk (132) no reference in the literature could be found of studies dealing with the chemical constituents in the blood of swine infected with cholera.

The results of the determinations have been arranged in four groups. The results recorded in Group 13 were from pigs which belonged to the Veterinary Research Department of Iowa State College. The results recorded in Group 14 were from pigs belonging to the Experiment Station of the United States Bureau of Animal Industry. Those recorded in Groups 15a and 15b were from pigs belonging to the Fort Dodge Serum Company, and were being utilized for the production of hog cholera virus.

The blood samples, procured from pigs belonging to the Veterinary Research Department, were drawn about eleven days after the pigs were injected with hog cholera virus. Those from the Experiment Station of the United States Bureau of Animal Industry were obtained seven days after virus injections and the blood samples from the Fort Dodge Serum Company were obtained six days after the pigs were injected with cholera virus. In each case the animals were on a starvation metabolism basis for from three to eight days, as they usually cease to eat on the second or third day following the onset of the disease of cholera.

Hog cholera represents a septicemia of swine, produced by a filtrable virus. The disease is always ushered in by an elevation of body tempera-

ture, and not until two or three days afterward are other perceptible symptoms shown. In severe acute cases the temperature remains at an approximately uniform height, between 40° and 42°C., until death. One of the first indications of the presence of the disease is impairment of the appetite, which rapidly develops into a complete anorexia.

### *Total Non-protein Nitrogen*

The results of total non-protein nitrogen determinations in the blood of the different groups of cholera-infected swine are recorded in table 25.

TABLE 25. *Maximum, minimum and mean values for total non-protein nitrogen in the different groups of cholera-infected swine*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. of blood		
			Maximum	Minimum	Mean
13	8	8	54.5	26.5	38.4
14	19	19	225.0	21.7	55.4
15a	8	8	73.1	27.7	46.1
15b	19	19	60.0	11.7	34.5

The average value for total non-protein nitrogen from 54 cholera-infected pigs was 43.8 milligrams per 100 c.c. of blood, ranging from 11.7 to 225 milligrams. Of these 54 cholera-infected pigs there were only two animals (numbers 12 and 21, original data) that had values over 75 milligrams of total non-protein nitrogen per 100 c.c. of blood. These animals had values of 225 and 202 milligrams, respectively. Both of these animals were in Group 14, which belonged to the Experiment Station of the United States Bureau of Animal Industry.

It seemed advisable for statistical study to consider an average value without these results. Such a value was found to be 37.0, ranging between 11.7 and 75.0 milligrams.

There is evidence of a higher non-protein nitrogen value in hog cholera-infected blood than in the blood of normal swine, which averaged 31.4 and ranged from 8.6 to 58 milligrams per 100 c.c.

The distribution of the value of total non-protein nitrogen are given in table 26.

TABLE 26. *Distribution of total non-protein nitrogen values in the blood of cholera-infected swine*

Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations	Mgms. of 100 c.c. of blood	Number of determinations	Percentage total no. determinations
Below 20	2	3.75	50-60	6	11.1
20-30	13	24.0	60-75	4	7.4
30-40	16	29.6	Above 75	2	3.75
40-50	11	20.4			

Seventy-four per cent of the total number of determinations of total non-protein nitrogen in the cholera-infected blood were between 20 and 50 milligrams per 100 c.c., whereas 76.6 per cent of the determinations on normal swine blood were between 20 and 40 milligrams per 100 c.c. Approximately 22 per cent of the determinations showed values over 50 milligrams per 100 c.c. of blood.

Roderick and Schalk (132) divided their results into two groups, one group with total non-protein nitrogen above 50 milligrams and another group in which values did not go above 50 milligrams per 100 c.c. of blood. They concluded that the group showing values for non-protein nitrogen above 50 milligrams per 100 c.c. of blood had a nitrogen retention, whereas the other group was within the range of normal values.

### *Urea Nitrogen*

The values obtained for urea nitrogen, the percentage of the total non-protein nitrogen represented by the urea nitrogen and the values for urea in the blood of cholera-infected swine are given in table 27.

TABLE 27. *Maximum, minimum and mean values for urea nitrogen in milligrams per 100 c.c. and percentage of total non-protein nitrogen and urea in the blood of different groups of cholera-infected swine*

Group number	Number of animals	Number of determinations	Urea N Mgms. per 100 c.c.			Percentage total non-protein nitrogen			Urea Mgms. per 100 c.c.		
			Maximum	Minimum	Mean	Maximum	Minimum	Mean	Maximum	Minimum	Mean
13	8	8	6.0	2.4	4.3	19.0	6.7	11.8	13.0	5.2	9.2
14	19	19	11.0	2.0	5.7	25.4	3.7	13.5	23.6	4.3	12.3
15a	8	8	5.0	2.2	3.2	11.3	5.4	7.0	10.7	4.7	6.9
15b	19	19	4.4	1.0	2.22	21.0	2.0	6.9	9.5	2.1	4.8

The average value for urea nitrogen in the cholera-infected blood obtained from 54 determinations on as many animals was 3.84 milligrams per 100 c.c., with a minimum of 1 milligram and a maximum of 11 milligrams per 100 c.c. The urea nitrogen averaged 10 per cent of the total non-protein nitrogen, ranging from 2.8 per cent to 26.4 per cent. The urea averaged 8.41 milligrams per 100 c.c. of blood, ranging from 2.1 to 23.6 milligrams.

The average value of 3.84 milligrams of urea nitrogen per 100 c.c. for the cholera-infected blood is lower than the average of 7.7 milligrams per 100 c.c. for the normal blood. The maximum of 11 milligrams per 100 c.c. for the cholera-infected blood is approximately one-half the value of the maximum for normal blood.

The data present evidence of a lower urea nitrogen value for cholera-infected pigs than for normal pigs. A lower value for urea nitrogen in

cholera-infected blood is difficult to explain inasmuch as there is evidence of an increase in the total non-protein nitrogen.

Out results showed lower values for urea nitrogen in the normal blood of swine than have been recorded by other investigators. Especially is this true in the group of normal pigs that were slaughtered at the abattoir of Iowa State College. Such low urea nitrogen values might result from the handling of the pigs during the twenty-four to thirty-six hours previous to slaughter. The still lower values of urea nitrogen in the cholera-infected blood may represent simply an exaggeration of the condition which may be the cause of the low values in normal swine (i. e., starvation), since the cholera-infected pigs may be assumed to be in a state of starvation metabolism for a much longer period of time than the abattoir group of normal pigs. In addition, such a condition would be augmented by a high body temperature in the case of the cholera-infected pigs.

Roderick and Schalk (132) obtained higher values for urea nitrogen in one group of their cholera-infected pigs than was found in their normal pigs.

The distribution of the urea nitrogen values found by us in cholera-infected blood is shown in table 28.

TABLE 28. *Distribution of urea nitrogen values in the blood of cholera-infected swine*

Mgms. per 100 c.c. of blood	Number of determinations	Percent- age total no. deter- minations	Mgms. per 100 c.c. of blood	Number of deter- minations	Percent- age total no. deter- minations
Below 2	9	16.6	6-7	6	11.1
2-3	11	20.4	7-8	1	1.9
3-4	10	18.5	8-9	0	0.0
4-5	8	14.8	9-10	3	5.5
5-6	5	9.3	Above 10	1	1.9

It will be noted that more than 90 per cent of the determinations of urea nitrogen in cholera-infected blood are below 7 milligrams per 100 c.c.

Wakeman and Morrell (151) made determinations of total non-protein nitrogen and the individual non-protein nitrogenous components (urea, amino-acid, ammonia, uric acid and creatinine) of the blood of normal "Macacus rhesus" monkeys and similar animals with experimentally produced yellow fever. They found that the uric acid, creatinine, rest nitrogen and ammonia were not constantly altered, although creatinine and rest nitrogen usually rose to a variable extent in the terminal stages of the disease.

The constituents which were chiefly affected were the urea and amino-acids. Amino-acid nitrogen rose rapidly in the terminal stages of the disease; urea nitrogen in some cases rose, but sometimes remained constant or fell. In any case the amino-acid nitrogen increased proportionately far more, and the urea nitrogen far less, than the total non-protein nitrogen. This was evidence that the power of the liver to deaminize amino-acids and to produce urea was greatly impaired or destroyed. This functional derangement became apparently only during the last hours of life.



Wakeman and Morrell (152) found that the amino-acids increased in both absolute amounts and in proportion to the non-protein nitrogen, while urea decreased in proportion to non-protein nitrogen. A few cases were found in which there was an absolute decrease in blood urea nitrogen. These changes were found to be terminal events and none of significance occurred during the early stages of the disease. The alterations observed were interpreted as resulting from a loss of liver function. No definite evidence of serious impairment of kidney function was observed, except a terminal anuria probably due to an extreme reduction of blood pressure.

### *Uric Acid*

The results for the uric acid determinations, uric acid nitrogen, and the percentage of the total non-protein nitrogen represented by the uric acid nitrogen in the blood of different groups of cholera-infected swine are given in table 29.

TABLE 29. *Maximum, minimum and mean values for uric acid and uric acid nitrogen in milligrams per 100 c.c., and percentage of total non-protein nitrogen in the blood of different groups of cholera-infected swine*

Group number	Number of animals	Number of determinations	Mgms. per 100			c.c. of blood			Uric acid nitrogen Percentage total non-protein nitrogen		
			Uric acid			Uric acid nitrogen					
			Maximum	Minimum	Mean	Maximum	Minimum	Mean	Maximum	Minimum	Mean
13	8	8	4.8	0.33	2.3	1.9	0.1	0.82	5.24	0.3	2.03
14	19	19	6.6	0.32	3.1	2.2	0.1	1.02	10.0	0.33	3.0
15a	8	8	9.2	0.26	1.53	3.0	0.08	0.5	7.1	0.2	1.17
15b	19	19	3.7	0.14	0.77	1.2	0.04	0.24	3.2	0.12	0.8

The average value for uric acid from 54 samples of cholera-infected blood was 1.92 milligrams per 100 c.c., ranging between 0.14 and 9.2 milligrams. For uric acid nitrogen the average value was 0.63 milligram per 100 c.c., ranging between 0.08 and 3.0 milligrams. The uric acid nitrogen averaged 1.8 per cent of the total non-protein nitrogen and varied between 0.12 and 10.0 per cent.

The average of 1.92 milligrams of uric acid in the blood of cholera-infected pigs is very close to the average of 1.95 milligrams for normal blood, although the range is somewhat greater in the cholera-infected blood.

The distribution of our uric acid values in cholera-infected blood is given in table 30.

Approximately 50 per cent of the uric acid values are between 0.2 and 0.7 milligrams per 100 c.c. of blood.

TABLE 30. *Distribution of uric acid values in the blood of cholera-infected swine*

Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations	Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations
Below 0.2	1	1.9	0.9-1.0	0	0.0
0.2-0.3	6	11.0	1.0-2.0	3	5.6
0.3-0.4	10	18.4	2.0-3.0	5	9.3
0.4-0.5	4	7.5	3.0-4.0	6	11.0
0.5-0.6	2	3.7	4.0-5.0	5	9.3
0.6-0.7	5	9.3	5.0-6.0	2	3.7
0.7-0.8	0	0.0	Above 6.0	3	5.6
0.8-0.9	2	3.7			

*Creatinine*

The results of the creatinine determinations and creatinine nitrogen in milligrams per 100 c.c. and percentage of total non-protein nitrogen for the blood of different groups of cholera-infected swine are reported in table 31.

TABLE 31. *Maximum, minimum and mean values for creatinine, creatinine nitrogen, and percentage of total non-protein nitrogen in different groups of cholera-infected blood*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. of blood						Creatinine N Percentage total non-protein nitrogen		
			Creatinine			Creatinine N					
			Maximum	Minimum	Mean	Maximum	Minimum	Mean	Maximum	Minimum	Mean
13	8	8	2.3	1.4	1.97	0.9	0.53	0.76	2.8	1.4	2.0
14	19	19	2.5	1.1	1.7	0.94	0.4	0.63	3.2	0.4	1.6
15a	8	8	4.1	2.0	2.7	1.6	0.8	1.02	4.3	1.36	2.73
15b	19	19	3.0	1.6	2.3	1.1	0.6	0.86	11.7	1.5	3.2

The average value for creatinine from 54 samples of cholera-infected blood was 2.21 milligrams per 100 c.c., ranging from 1.7 to 4.1 milligrams. The creatinine nitrogen averaged 0.79 milligrams per 100 c.c. and varied between 0.4 and 1.6 milligrams. The creatinine nitrogen averaged 2.36 per cent of the total non-protein nitrogen, ranging from 0.4 to 11.7 per cent.

The average of 2.21 milligrams of creatinine per 100 c.c. of cholera-infected blood is higher than the average of 1.6 found in the normal blood of swine.

The distribution of the creatinine values in cholera-infected blood is given in table 32.

TABLE 32. *Distribution of creatinine values in cholera-infected blood*

Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations	Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations
Below 1.5	5	9.3	2.5-3.0	7	12.9
1.5-2.0	14	25.8	3.0-3.5	3	5.7
2.0-2.5	24	44.4	Above 3.5	1	1.9

Approximately 70 per cent of the total number of determinations gave a value for creatinine between 1.5 and 2.5 milligrams per 100 c.c. of blood.

### *Creatine Plus Creatinine*

The results of creatine plus creatinine determinations in different groups of cholera-infected blood are recorded in table 33.

TABLE 33. *Maximum, minimum and mean values for creatine plus creatinine in the blood of different groups of cholera-infected swine*

Group Number	Number of Animals	Number of determinations	Mgms. per 100 c.c. of blood		
			Maximum	Minimum	Mean
13	8	8	15.0	4.1	8.2
14	19	19	10.0	2.0	5.6
15a	8	8	10.0	2.9	6.5
15b	19	19	7.7	4.4	6.4

The average value for creatine plus creatinine of 54 cholera-infected blood samples was 6.3 milligrams per 100 c.c., with a minimum of 2.0 and a maximum of 15.0 milligrams.

The average value for creatine plus creatinine in cholera-infected blood is slightly higher than in normal blood and the variation is somewhat greater in the former.

The distribution of our values of creatine plus creatinine in cholera-infected blood are shown in table 34.

TABLE 34. *Distribution of creatine plus creatinine values in cholera-infected blood*

Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations	Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations
2-3	3	5.6	7-8	9	16.5
3-4	1	1.9	8-9	2	3.7
4-5	11	20.4	9-10 incl.	2	3.7
5-6	4	7.5	Above 10	3	5.6
6-7	19	35.1			

Sixty-three per cent of the values for creatine plus creatinine are between 4.0 and 7.0 milligrams per 100 c.c. of blood.

### *Sugar*

The results of the blood sugar determinations in the blood of the various groups of cholera-infected swine are shown in table 35.

TABLE 35. *Maximum, minimum and mean values for blood sugar in various groups of cholera-infected blood*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. of blood		
			Maximum	Minimum	Mean
13	7	7	200	80	107
14	19	19	97	28	54
15a	8	8	90	43	65
15b	19	19	133	40	78

The average value for blood sugar from 53 samples of cholera-infected blood was 71 milligrams per 100 c.c., with a minimum of 29 milligrams and a maximum of 200 milligrams. These values are definitely lower in the cholera-infected blood than in the normal blood.

The distribution of the sugar values obtained by us in cholera-infected blood is shown in table 36.

TABLE 36. *Distribution of sugar values in cholera-infected blood*

Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations	Mgms. per 100 c.c. of blood	Number of determinations	Percentage total no. determinations
Below 30	2	3.7	80-90	6	11.3
30-40	3	5.6	90-100	4	7.5
40-50	5	9.4	100-110	2	3.7
50-60	6	11.3	110-120	3	5.6
60-70	16	30.7	Above 120	2	3.7
70-80	4	7.5			

Eighty-seven per cent of the determinations gave values under 100 milligrams per 100 c.c. of blood. Approximately 50 per cent of the determinations gave values between 60 and 90 milligrams of sugar per 100 c.c. of blood.

### *Inorganic Phosphorus*

The results of the determinations for inorganic phosphorus in the blood of various groups of cholera-infected swine are reported in table 37.



TABLE 37. *Maximum, minimum and mean values for inorganic phosphorus in the blood of various groups of cholera-infected swine*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. of serum		
			Maximum	Minimum	Mean
13	8	8	11.0	5.0	7.2
14	19	19	14.0	4.6	7.4
15a	8	8	9.0	5.1	6.6
15b	19	19	6.4	4.0	5.0

The average of 54 determinations of inorganic phosphorus gave a value of 6.44 milligrams per 100 c.c. of serum, with a minimum of 4.0 and a maximum of 14.0 milligrams.

The distribution of the inorganic phosphorus values in cholera-infected blood is shown in table 38.

TABLE 38. *Distribution of the inorganic phosphorus values in the blood of cholera-infected swine*

Mgms. per 100 c.c. of serum	Number of determinations	Percentage total no. determinations	Mgms. per 100 c.c. of serum	Number of determinations	Percentage total no. determinations
4-5	12	22.1	8-9	4	7.4
5-6	22	41.0	9-10	4	7.4
6-7	6	11.1	10-11	3	5.6
7-8	1	1.8	Above 11	2	3.6

The average value for inorganic phosphorus in cholera-infected blood is lower than that for normal blood. The lower value in cholera-infected blood may be due to less exercise and struggling on the part of the cholera pigs in the procedure of drawing the blood at the time of killing.

Approximately 75 per cent of the determinations gave values for inorganic phosphorus between 4.0 and 7.0 milligrams per 100 c.c. of serum.

### Calcium

The calcium value in the blood of various groups of cholera-infected swine are shown in table 39.

TABLE 39. *Maximum, minimum and mean calcium values in the blood of various groups of cholera-infected swine*

Group number	Number of animals	Number of determinations	Mgms. per 100 c.c. of serum		
			Maximum	Minimum	Mean
13	8	8	19	13	15
14	19	19	20	6	11.4
15a	8	8	22	14	17.2
15b	19	19	24	9	14.7

The average value for calcium from 54 samples of cholera-infected blood was 14 milligrams per 100 c.c. of serum. The values ranged between a minimum of 6 milligrams and a maximum of 24 milligrams.

The average value for the calcium concentration in cholera-infected blood is higher than that obtained for normal pig blood.

The distribution of the calcium values in cholera-infected blood is shown in table 40.

TABLE 40. *Distribution of the calcium values in cholera-infected blood of swine*

Mgms. per 100 c.c. of serum	Number of determina- tions	Percentage total no. determina- tions	Mgms. per 100 c.c. of serum	Number of determina- tions	Percentage total no. determina- tions
Below 9	3	5.65	15-16	5	9.4
9-10	2	3.6	16-17	3	5.65
10-11	7	12.8	17-18	5	9.4
11-12	2	3.6	18-19	2	11.3
12-13	7	12.8	19-20	6	3.6
13-14	8	15.0	Above 20	2	3.6
14-15	2	3.6			

Approximately 48 per cent of the total number of determinations for calcium were between 9 and 14 milligrams per 100 c.c. of serum.

#### NORMAL AND CHOLERA-INFECTED BLOOD COMPARED

The data obtained from these studies were subjected to statistical analysis. The means of these constituents in both the normal and in the hog cholera-infected blood, with the probable error of each mean are shown in table 41. The table also shows the mean differences, the probable errors of the mean differences, and the ratio of each mean difference to its probable error. The ratios which are significant are indicated by italics.

The mean value for total non-protein nitrogen in the cholera-infected blood was obtained by substituting arbitrary values for numbers 12 and 21 (group 14) that would lie in the range between 11.7 and 75.0.

In making these calculations the usual criterion of a significant difference was used, that is, if the mean difference is equal to or greater than three times the probable error of the mean difference the ratio is significant.

It will be seen in table 41 that there is a significant mean difference in favor of cholera-infected blood in the concentration of total non-protein nitrogen. Roderick and Schalk (132) found the blood of cholera-infected pigs, drawn at various stages of the disease, was often normal as far as the total non-protein nitrogen was concerned. Some of the samples of blood showed moderate evidences of nitrogenous retention. Our results indicate that certain cases showed markedly increased total non-protein nitrogen values.

The mean urea nitrogen value is significant in favor of the normal blood. Roderick and Schalk (132) found an increase in urea nitrogen in the blood of one group of cholera-infected pigs, whereas in the blood of an-

TABLE 41. *Comparison of certain chemical constituents in normal and cholera-infected blood of swine*

Constituents	Normal swine		Cholera swine	Mean difference (-) signifies mean difference is in favor of chol- era blood	Ratio
	Number of deter- minations	Means for normal blood	Means for cholera blood		
Total non-protein nitrogen.....	74	Mgm. per 100 c.c. 31.89 $\pm$ 0.74	Mgm. per 100 c.c. 37.02 $\pm$ 1.234	— 5.13 $\pm$ 1.44	3.5
Urea nitrogen .....	74	7.42 $\pm$ 0.43	3.84 $\pm$ 0.22	3.57 $\pm$ .48	7.4
Uric acid .....	74	1.94 $\pm$ 0.10	1.92 $\pm$ 0.17	0.01 $\pm$ .24	0.07
Creatinine .....	74	1.69 $\pm$ 0.26	2.21 $\pm$ 0.09	— 0.51 $\pm$ 1.8	0.27
Sugar .....	62	125.95 $\pm$ 9.43	71.38 $\pm$ 2.68	54.56 $\pm$ 9.8	5.56
Inorganic phosphorus .....	62	7.22 $\pm$ 0.15	6.4 $\pm$ 0.2	0.82 $\pm$ 0.25	3.2
Calcium .....	62	12.39 $\pm$ 0.28	14.01 $\pm$ 0.36	— 1.62 $\pm$ 0.46	3.49

other group the average value for this constituent remained about the same as the value they obtained for normal swine blood. The lower mean values for urea nitrogen in cholera-infected blood which our data show may represent an exaggeration of the condition causing low urea nitrogen values in normal swine blood.

The mean value for uric acid is approximately the same for both kinds of blood.

The mean value for creatinine is slightly higher for cholera-infected blood than it is for normal blood, but the ratio of the mean difference to its probable error is not great enough for the difference to be considered significant. In other similar studies the mean difference might readily be higher for normal blood.

There is a significant decrease in the mean sugar and phosphorus values, whereas the mean calcium value shows a significant increase in cholera-infected blood.

The prolonged starvation metabolism may account for the decreased average value of sugar in cholera-infected blood.

The decreased mean value of inorganic phosphorus in cholera-infected blood may be due in part to less resistance and struggling on the part of the pigs, in the operation of obtaining of blood as the animals are killed, and in part, the decreased values may be a factor of the disease.

The increased mean calcium value in cholera-infected blood may be associated with the disease.

The correlation coefficients between non-protein nitrogen, urea nitrogen, uric acid and creatinine in the blood of normal swine are recorded in table 42.

TABLE 42. *Correlations between certain chemical constituents in the blood of 74 normal swine*

	Urea nitrogen	Uric acid	Creatinine
Total non-protein nitrogen	0.18	—0.02	—0.13
Urea nitrogen		—0.37	—0.35
Uric acid			—0.04

According to Fisher's table Va (41) the correlation coefficient must be 0.2319 or greater to be significant when seventy-four animals are used. Thus it is seen in table 42 that there are but two significant correlations; one of —0.37 between uric acid and urea nitrogen and the other of —0.35 between creatinine and urea nitrogen. A negative correlation between two variables indicates that an increase in one is accompanied by a decrease in the other.

In table 43 the correlation coefficients between non-protein nitrogen, urea nitrogen, uric acid and creatinine in cholera-infected blood of swine are recorded.

According to Fisher's table Va (41) when 54 animals are used the correlation coefficient must be 0.2732 or greater to be significant.

A study of table 43 shows that in cholera-infected blood there is a significant correlation of 0.3 between urea nitrogen and uric acid, whereas



TABLE 43. *Correlations between certain chemical constituents in the blood of 54 cholera-infected swine*

	Urea nitrogen	Uric acid	Creatinine
Total non-protein nitrogen	0.19	0.01	0.07
Urea nitrogen		0.3	0.04
Uric acid			0.25

in normal blood there is a significant negative correlation between the two constituents.

The correlation between uric acid and creatinine of  $-0.25$  is just below the border line of the test for significance established by R. A. Fisher. However, such a coefficient is suggestive of a negative correlation between the two variables.

The correlation coefficients between sugar, inorganic phosphorus and calcium in the blood of normal swine are given in table 44.

TABLE 44. *Correlations between sugar, inorganic phosphorus and calcium in the blood of 62 normal swine*

	Inorganic phosphorus	Calcium
Sugar	0.57	$-0.14$
Inorganic phosphorus		$-0.09$

Fisher's table Va (41) states that when 62 animals are used a correlation coefficient of 0.25 is significant and that a correlation coefficient of 0.32 is highly significant.

The only significant correlation in table 44 is that of 0.57 between sugar and phosphorus.

The correlation coefficients between sugar, phosphorus and calcium in the blood of cholera-infected swine are recorded in table 45.

TABLE 45. *Correlations between sugar, inorganic phosphorus and calcium in the blood of 54 cholera-infected swine*

	Inorganic phosphorus	Calcium
Sugar	$-0.22$	$-0.34$
Inorganic phosphorus		$-0.22$

Referring to Fisher's table Va (41) the correlation coefficient must be 0.27 or greater to be significant when 54 animals are used.

The only significant correlation presented in table 45 is that of 0.34 between sugar and calcium. However, the negative correlation of  $-0.22$  between sugar and inorganic phosphorus, and the negative correlation of

—0.22 between inorganic phosphorus and calcium, are on the border line and suggestive of significance. In other words sugar and inorganic phosphorus show a tendency to vary in opposite directions as do also inorganic phosphorus and calcium in cholera-infected blood.

#### SUMMARY

A review of the literature dealing with the methods used in practical chemical analysis of blood is given, as well as a review of literature concerning the normal concentration of the chemical constituents in human blood and the blood of domestic animals.

The literature, which is limited, dealing with the chemical constituents of the normal blood of swine is noted. One reference only was found that considered the chemical constituents of the blood of swine infected with hog cholera.

The results of determinations of total non-protein nitrogen and components (urea, uric acid, creatinine, creatine plus creatinine), sugar, phosphorus and calcium in the blood of normal swine and in the blood of cholera-infected swine are reported.

The results of 107 determinations of total non-protein nitrogen in the blood of 98 pigs gave an average value of 31.4 milligrams per 100 c.c., which is in fair agreement with the average values reported by other investigators.

The average value for urea nitrogen from 81 determinations of normal swine blood from 73 pigs was 7.7 milligrams per 100 c.c. This average is lower than average values recorded by other investigators for normal swine blood. (137, 58.)

The average value of 1.95 milligrams per 100 c.c. for uric acid from 78 determinations of normal swine blood from 71 pigs agrees with average values recorded for other species. Several investigators stated that they could find no uric acid in swine blood.

Creatinine averaged 1.6 milligrams per 100 c.c. for 106 determinations of normal swine blood from 97 pigs, which is in agreement with other investigators.

The average value of 128 milligrams of sugar per 100 c.c. for 72 determinations on normal swine blood from 65 pigs is higher than the average recorded for swine by other investigators, but lower than some averages recorded for other species.

Inorganic phosphorus averaged 7.26 milligrams per 100 c.c. of normal swine serum for 69 determinations from the blood of 61 pigs. This is higher than the average recorded for other species.

An average of 12.39 milligrams of calcium per 100 c.c. of normal swine serum was obtained from 71 determinations from the blood of 63 pigs.

Fifty-four determinations on cholera-infected blood from the same number of pigs gave the following averages: total non-protein nitrogen, 37 milligrams per 100 c.c. of blood; urea nitrogen, 3.84 milligrams per 100 c.c. of blood; uric acid, 1.92 milligrams per 100 c.c. of blood; creatinine, 2.21 milligrams per 100 c.c. of blood; creatine plus creatinine, 6.3 milligrams per 100 c.c. of blood; sugar, 71 milligrams per 100 c.c. of blood; inorganic phosphorus, 6.4 milligrams per 100 c.c. of serum; and calcium, 14 milligrams per 100 c.c. of serum.

Seventy-four determinations on normal swine blood of total non-protein nitrogen, urea nitrogen, creatinine, and 62 determinations of sugar, inorganic phosphorus and calcium in normal blood and 54 determinations of each of these constituents in cholera-infected blood were used for statistical analysis.

There was found to be a significant mean difference for total non-protein nitrogen in favor of cholera-infected blood. A significant mean difference for urea nitrogen in favor of normal blood was found.

The mean value for uric acid was practically the same for both kinds of blood.

The mean value for creatinine was slightly higher for cholera-infected blood than for normal swine blood. The creatine plus creatinine mean value is also higher for cholera-infected blood than for normal swine blood.

There was a significant decrease in the sugar, and phosphorus mean values for cholera-infected blood as compared with normal blood.

The mean value for calcium showed a significant increase for cholera-infected blood as compared with the normal blood of swine.

The data accumulated from the observations on normal and cholera-infected blood were studied from the group standpoint. The methods used were recognized standard laboratory procedures.

#### CONCLUSIONS

The meager literature relative to the composition of the blood of animals offers only limited opportunity for comparison of the data recorded in this paper with that of similar investigations.

The concentration of total non-protein nitrogen in the normal blood of swine was found to have a somewhat wider variation than that recorded for other species. The average value of 31.4 milligrams of total non-protein nitrogen per 100 c.c. of blood agrees closely with the average value reported for other species. In some cases of hog cholera the total non-protein nitrogen may increase to a very high level, while in other and perhaps most cases of hog cholera, the total non-protein nitrogen concentration of the blood is not disturbed.

Lower values were found for urea nitrogen in normal swine blood than were recorded by other investigators. These lower values, however, seemed for the most part restricted to the pigs slaughtered at the Iowa State College abattoir. An explanation for these low urea nitrogen values may be the withholding of food and water from the animals for twenty-four to thirty-six hours previous to slaughter. The still lower values for urea nitrogen found in the cholera-infected pigs may represent a prolongation of a starvation metabolism. There is some evidence that certain physiologic properties of the liver were deranged, especially the properties relative to urea formation, and possibly to uric acid destruction. Perhaps the histologic structure of the pig's liver lends itself to relatively easy derangement. Such a condition may be augmented in the disease of hog cholera.

Several instances of extremely high sugar values in normal swine blood were encountered. It is possible that the same factors that result in low urea concentrations may be responsible for the abnormal blood sugar values.

Only limited evidence was obtained which would indicate renal impairment in hog cholera. The few instances of high total non-protein nitrogen

values in the cholera-infected blood would indicate that there were nitrogen retentions in some cases. The slightly higher average value for creatinine in cholera-infected blood might suggest some renal impairment.

A study of these data reveals the fact that in general most of the chemical constituents are subject to a wider variation in swine blood than in human blood.

It is evident that a study of the composition of normal swine blood and cholera-infected blood of this nature is merely introductory to great possibilities of future investigations.

#### LITERATURE CITED

1. ABERHALDEN, E.  
1898. Zur quantitativen vergleichenden Analyse des Blutes. *Ztschr. Physiol. Chem.*, **25**:65-115.
2. ALLERS, R. A., AND S. BONDI  
1907. Über das Verhalten des Calciums in Blute bei experimentellen Säure Vergiftung. *Biochem. Ztschr.*, **6**:366-378.
3. ANDERSON, A. K., H. E. GAYLEY AND A. D. PRATT  
1930. Studies on the chemical composition of bovine blood. *Jour. Dairy Science*, **13**:336-348.
4. —————, H. E. HONEYWELL, A. C. SANTY AND S. PEDERSON  
1930. The composition of normal rat blood. *J. Biol. Chem.*, **86**:157-160.
5. —————, G. H.  
1924. The calcium and phosphorus content of the blood in normal and rachitic children. *Brit. J. Child. Dis.*, **21**:43-48.
6. AWDEJEWA, M. S., E. L. PROWATOROWA, N. G. SAWITSCH AND E. L. THAL  
1927. Schwankungen des Blutzuckergehaltes beim Rind. *Biochem. Ztschr.*, **187**:369-376.
7. BELL, R. D., AND E. A. DOISY  
1920. Rapid colorimetric methods for the determination of phosphorus in urine and blood. *J. Biol. Chem.*, **44**:55-67.
8. BENEDICT, S. R.  
1925. The determination of blood sugar. *J. Biol. Chem.*, **64**:207-219.
9. —————  
1928. The determination of blood sugar. *J. Biol. Chem.*, **76**:457-470.
10. —————  
1922. The determination of uric acid in blood. *J. Biol. Chem.*, **51**:187-207.
11. —————  
1918. A modification of the Lewis-Benedict method for the determination of sugar in the blood. *J. Biol. Chem.*, **34**:203-207.
12. —————  
1915. On the colorimetric determination of uric acid in the blood. *J. Biol. Chem.*, **20**:629-631.
13. —————  
1915. Studies in uric acid metabolism. I. On the uric acid in ox and chicken blood. *J. Biol. Chem.*, **20**:633-640.
14. —————  
1916. Uric acid in relation to metabolism. *J. Lab. and Clin. Med.*, **2**:1-15.



15. ———, AND E. A. HITCHCOCK  
1915. On the colorimetric estimation of uric acid in blood. *J. Biol. Chem.*, **20**:619-627.
16. ———, E. B. NEWTON AND J. A. BEHRE  
1926. A new sulphur containing compound. *J. Biol. Chem.*, **67**:267-277.
17. ———, AND R. C. THEIS  
1924. A modification of the molybdc method for the determination of inorganic phosphorus in serum. *J. Biol. Chem.*, **61**:63-66.
18. BERNARD, CLAUDE  
1849. De l' origine du sucre. *Mem. Soc. de Biol.*, **1**:121-133.
19. BIESTER, H. E.  
1925. Diabetes in a pig showing pancreatic lesions. *J. Am. Vet. Med. Assoc.*, **67**:99-109.
20. BLOOR, W. R.  
1918. The distribution of phosphoric acid in normal human blood. *J. Biol. Chem.*, **36**:49-57.
21. ———.  
1918. Methods for determination of phosphoric acid in small amounts of blood. *J. Biol. Chem.*, **36**:33-48.
22. BOLLMAN, J. L., F. C. MANN AND T. B. MAGATH  
1925. Studies on the physiology of the liver. *Am. J. Physiol.*, **72**:629-646.
23. ———.  
1926. Studies on the physiology of the liver. XV. Effect of total removal of the liver on deamination. *Am. J. Physiol.*, **78**:258-269.
24. BRIGGS, A. P.  
1922. A modification of the Bell-Doisy phosphate method. *J. Biol. Chem.*, **53**:13-16.
25. ———.  
1924. Some applications of the colorimetric phosphate method. *J. Biol. Chem.*, **59**:255-264.
26. BROcq-ROUSSEAU, D., G. ROUSSEL AND GALLOT  
1929. Sur l' urée du sang du cheval. *Sang*, **3**:173-177.
27. BROWN, H., AND G. W. RAIZISS  
1922. The estimation of uric acid in blood. *J. Lab. and Clin. Med.*, **8**:129-134.
28. ———, W. H.  
1928. Calcium and inorganic phosphorus in the blood of rabbits. I. Results of repeated observations in normal rabbits. II. Results of single observations on normal rabbits from recently acquired stock. *J. Exp. Med.*, **47**:539-576.
29. BUELL, M. V.  
1923. On the phosphorus compounds in normal human blood. *J. Biol. Chem.*, **56**:97-107.
30. ———.  
1919. Studies on blood regeneration. I. Effect of hemorrhage on alkaline reserve. *J. Biol. Chem.*, **40**:29-61.
31. BULMER, F. M. R., A. EAGLES AND G. HUNTER  
1925. Uric acid determinations in blood. *J. Biol. Chem.*, **63**:17-35.
32. BUTKA, H. E., AND MEISNER, F. E.  
1925. Technical improvements in urea distillation by the Folin-Wu method. *J. Lab. and Clin. Med.*, **10**:937-938.

33. BYROM, F. B., AND H. D. KAY  
1927. Blood phosphorus in health and disease. Changes in phosphorus compounds in renal disease. *Brit. J. Exp. Path.*, **8**:429-436.
34. CARLSON, A. J., AND F. M. DRENNAN  
1912. A note on the sugar tolerance in the pig. *J. Biol. Chem.*, **13**:465-468.
35. CHANUTIN, A., AND H. SILVETTE  
1928. The influence of fasting and creatine feeding upon the creatine content of the tissues and blood of the white rat. *J. Biol. Chem.*, **80**:589-608.
36. CHASE, A. F., AND MYERS, V. C.  
1916. The value of recent laboratory tests in the diagnosis and treatment of nephritis. *J. Am. Med. Assoc.*, **67**:929-932.
37. CLARK, E. P., AND J. B. COLLIP  
1925. A study of the Tisdall method for determination of blood serum calcium with a suggested modification. *J. Biol. Chem.*, **63**:461-464.
38. ———, G. W.  
1921. The micro-determination of calcium in whole blood, plasma and serum by direct precipitation. *J. Biol. Chem.*, **49**:487-517.
39. COOPER, F. B.  
1925. Contributing cause of turbidity of nesslerized solutions in determination of urea in whole blood. *J. Lab. and Clin. Med.*, **10**:1012.
40. CULHANE, K.  
1927. Variations in serum-calcium of rabbits. *Biochem. J.*, **21**:1015-1023.
41. DANIELOPOLU, D., AND M. MAXIM  
1928. Über die Blutgehalts Veränderungen an Kalium, Calcium und Cholin während des Angina-Pectoris-Anfalles. *Klin. Wehnschr.*, **7**:1466-1467.
42. DAVIS, A. R., AND S. R. BENEDICT  
1921. A crystalline uric acid compound in beef blood. *J. Biol. Chem.*, **46**:v-vi.
43. DENIS, W., AND A. S. MINOT  
1920. A study of phosphate retention from the standpoint of blood analysis. *Arch. Int. Med.*, **26**:99-104.
44. DE WESSELOW, O. L. V.  
1923. The immediate prognosis in nephritis with remarks on uraemia. *Lancet*, **205**:163-165.
45. FEINBLATT, H. M.  
1923. Creatininemia, based upon a study of 1500 blood chemical analyses. *Am. J. Med. Sci.*, **166**:249-266.
46. ———.  
1923. Hyperglycemia, based upon a study of 2000 blood chemical analyses. *J. Lab. and Clin. Med.*, **8**:500-505.
47. FISHER, R. A.  
1930. Statistical methods for research workers. Oliver and Boyd, London.
- 47a. FISKE, C. H., AND Y. SUBBAROW  
1925. The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66**:375-400.
48. FOLIN, O.  
1926. The determination of sugar in blood and normal urine. *J. Biol. Chem.*, **67**:357-370.
49. ———.  
1929. Laboratory manual of biological chemistry. Appleton, New York. Pp. 237-243.

50. \_\_\_\_\_  
1922. Non-protein nitrogen of blood in health and disease. *Physiol. Rev.*, 2:460-478.
51. \_\_\_\_\_  
1914. On the determination of creatinine and creatine in blood, milk and tissues. *J. Biol. Chem.*, 17:475-481.
52. \_\_\_\_\_  
1922. A system of blood analysis. Supplement. IV. A revision of the method for determining uric acid. *J. Biol. Chem.*, 54:153-170.
53. \_\_\_\_\_ AND H. BERGLUND  
1922. The retention and distribution of amino acid with special reference to the urea formation. *J. Biol. Chem.*, 51:395-418.
54. \_\_\_\_\_ AND W. DENIS  
1913. New colorimetric method for the determination of uric acid in the blood. *J. Biol. Chem.*, 13:469-475.
55. \_\_\_\_\_ AND \_\_\_\_\_  
1913. New methods for the determination of total non-protein nitrogen, urea and ammonia in the blood. *J. Biol. Chem.*, 11:527-536.
56. \_\_\_\_\_ AND \_\_\_\_\_  
1916. Nitrogen determinations by direct nesslerization. II. Non-protein nitrogen in blood. *J. Biol. Chem.*, 26:491-496.
57. \_\_\_\_\_ AND \_\_\_\_\_  
1914. On the creatinine and creatine content of the blood. *J. Biol. Chem.*, 17:487-491.
58. \_\_\_\_\_ AND \_\_\_\_\_  
1915. Protein metabolism from the standpoint of blood and tissue analysis. VI. On uric acid, urea and total non-protein nitrogen in human blood. *J. Biol. Chem.*, 20:629-631.
59. \_\_\_\_\_ AND A. B. MACCOLLUM  
1912. On the blue color reaction of phosphotungstic acid with uric acid and other substances. *J. Biol. Chem.*, 11:265-266.
60. \_\_\_\_\_ AND J. L. MORRIS  
1913. Normal protein metabolism of the rat. *J. Biol. Chem.*, 14:509-515.
61. \_\_\_\_\_ AND H. WU  
1919. A system of blood analysis. *J. Biol. Chem.*, 38:81-110.
62. \_\_\_\_\_ AND \_\_\_\_\_  
1920. A system of blood analysis. Supp. I. A simplified and improved method for the determination of sugar. *J. Biol. Chem.*, 41:367-374.
63. FOSTER, G. L.  
1923. Studies on carbohydrate metabolism. I. Some comparisons of blood sugar concentrations in venous blood and in finger blood. *J. Biol. Chem.*, 55:291-301.
64. GARROD, A. B.  
1848. Observations on certain pathological conditions of the blood and urine in gout, rheumatism and Bright's disease. *Med. chir. Trans.*, 31:83-87.
65. GETTLER, A. O., AND W. BAKER  
1916. Chemical and physical analysis of blood in 30 normal cases. *J. Biol. Chem.*, 25:211-222.
66. GREENWALD, I.  
1915. The estimation of lipid and acid soluble phosphorus in small amounts of serum. *J. Biol. Chem.*, 21:29-36.

67. ————. 1918. The estimation of non-protein nitrogen in blood. *J. Biol. Chem.*, **34**:97-118.
68. ———— AND G. MCGUIRE 1918. The estimation of creatinine and creatine in blood. *J. Biol. Chem.*, **34**:103-118.
69. GREISHEIMER, E. M. 1928. Blood sugar and irritability. *Minnesota Medicine*, **11**:239-244.
70. GEUSKIN, B. 1924. A new and shorter method for the determination of urea and sugar in the blood. *J. Lab. and Clin. Med.*, **10**:233-236.
71. HADEN, R. L., AND T. G. ORR 1923. Changes in the blood of the dog after intestinal obstruction. *J. Exp. Med.*, **37**:365-375.
72. ———— AND ————. 1923. Chemical changes in the blood of the dog after obstruction of the oesophagus and of the cardiac end of the stomach. *J. Exp. Med.*, **38**:477-485.
73. ———— AND ————. 1923. Chemical changes in the blood after pyloric obstruction. *J. Exp. Med.*, **37**:377-381.
74. ———— AND ————. 1925. Chemical findings in the blood of the normal dog. *J. Biol. Chem.*, **65**:479-481.
75. HAGEDORN, H. C., AND B. N. JENSEN 1923. Microbestimmung des Blutzuckers sur mittels Ferricyanid. *Biochem. Ztschr.*, **135**:46-58.
76. HALVERSON, J. O., H. K. MOHLER AND O. BERGEIM 1917. The calcium content of blood serum in certain pathological conditions. *J. Biol. Chem.*, **32**:171-179.
77. HAMMETT, F. S. 1920. Studies of variations in the chemical composition of human blood. *J. Biol. Chem.*, **41**:599-614.
78. HARDING, V. T., K. D. ALLIN, B. A. EAGLES AND H. B. VAN WYCK 1925. The effect of high fat diets on the content of uric acid in the blood. *J. Biol. Chem.*, **63**:37-53.
79. HARNES, A. R. 1928. Biometry of calcium, inorganic phosphorus, cholesterol and lipid phosphorus in the blood of rabbits. I. Normal animals from recently acquired stock. *J. Exp. Med.*, **48**:549-565.
- 79a. ————. 1929. Biometry of calcium, inorganic phosphorus, cholesterol and lipid phosphorus in the blood of rabbits. II. Repeated observations on normal animals. *J. Exp. Med.*, **49**:287-301.
80. HART, E. B., H. STERNBOCK, C. A. HOPPERT AND R. M. BETHKE 1922. Dietary factors influencing calcium assimilation. III. The comparative efficiency of timothy hay, alfalfa hay and timothy hay plus calcium phosphate (steamed bone meal) in maintaining calcium and phosphorus equilibrium in milking cows. *J. Biol. Chem.*, **54**:75-89.
81. HAWK, P. B., AND O. BERGEIM 1927. *Practical Physiological Chemistry*. Blakiston, Philadelphia.



82. HAYDEN, C. E., AND P. A. FISH  
1928. The normal blood of some domesticated animals. *Cornell Veterinarian*, **18**:197-203.
83. ——— AND M. TUBANGUI  
1919. Studies in the normal blood of domestic animals. *Ann. Report, N. Y. State Vet. College*, **20**:181-192.
84. HENRY, W. A., AND F. B. MORRISON  
1923. *Feeds and Feeding*. The Henry-Morrison Co., Madison.
85. HESS, J. H., J. K. CALVIN, C. C. WANG AND A. FELTCHER  
1923. Calcium and phosphorus determinations in blood plasma in rickets and tetany. *Am. J. Dis. Child.*, **26**:271-279.
86. HEWITT, E. A.  
1930. The blood sugar level of the bovine. *J. Am. Vet. Med. Assoc.*, **77**:362-377.
87. HILL, R. M.  
1928. Effect of administration of creatine on the blood sugar. *Proc. Am. Soc. Biol. Chem.* **22**. IV, 1928. *J. Biol. Chem.*, **78**.
88. HOLBROOK, W. J., AND H. D. HASKINS  
1926. Blood uric acid in nephritis. *J. Lab. and Clin. Med.*, **12**:10-15.
89. HOLT, R. L., AND H. R. REYNOLDS  
1924. Blood chemistry of the horse. *J. Am. Vet. Med. Assoc.*, **65**:732-736.
90. HORVATH, A. A., AND R. B. LITTLE  
1928. Studies in blood composition of animals under pathological conditions. I. Broncho-pneumonia in cows. *J. Clin. Inves.*, **5**:541-546.
91. HOWLAND, J.  
1923. The etiology and the pathogenicity of rickets. *Medicine*, **2**:349-374.
92. ——— AND B. KRAMER  
1921. Calcium and phosphorus in the serum in relation to rickets. *Am. J. Dis. Child.*, **22**:105-119.
93. HUBBARD, R. S.  
1927. Urea and creatinine concentrations. A statistical study. *Proc. Soc. Exp. Biol. and Med.*, **25**:20-22.
94. HUNTER, A., AND W. R. CAMPBELL  
1918. Amount and distribution of creatinine in normal human blood. *J. Biol. Chem.*, **33**:169-191.
95. JAFFÉ, M.  
1886. Über den Niederschlag welchen Pikrinsäure in normalen Harn erzeugt und über eine neue Reaction des Kreatinins. *Ztschr. Physiol. Chem.*, **10**:391-400.
96. JANSEN, B. C. P.  
1915. The function of the liver in urea formation from amino acids. *J. Biol. Chem.*, **21**:557-561.
97. JOHN, H. J.  
1928. Analyses of 10,368 synchronous sugar urea determinations. *Med. J. and Rec.*, **128**:498-503.
98. JOHNSON, F. P.  
1918. The isolation, shape, size and number of the lobules of the pig's liver. *Am. J. Anat.*, **23**:273-283.
99. ———, S. L.  
1924. Technical improvement in determination of blood urea by the Folin-Wu method. *J. Lab. and Clin. Med.*, **9**:860-863.

100. KARR, W. G.  
1924. A method for the determination of blood urea nitrogen. *J. Lab. and Clin. Med.*, **9**:329-333.
101. KINGSBURY, F. B., AND J. P. SEDGWICK,  
1917. The uric acid content of the blood of the new-born. *J. Biol. Chem.*, **31**:261-268.
102. KOCH, F. C., AND T. L. McMEekin  
1924. A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. *J. Am. Chem. Soc.*, **46**:2066-2069.
103. KOECHIG, I.  
1923. The calcium content of the blood in pathologic conditions. *J. Lab. and Clin. Med.*, **9**:679-685.
104. KRAMER, B., AND F. F. TISDALL  
1922. The distribution of sodium, potassium, calcium and magnesium between the corpuscles and serum of human blood. *J. Biol. Chem.*, **53**:241-252.
105. ——— AND ———  
1921. A simple technique for the determination of calcium and magnesium in small amounts of serum. *J. Biol. Chem.*, **47**:475-481.
106. LEIBOFF, S. L., AND I. S. WITCHELL  
1929. Use of sodium citrate as anticoagulant in chemical examination of blood. *J. Lab. and Clin. Med.*, **14**:1094-1096.
107. LEWIS, R. C., AND S. R. BENEDICT  
1915. A method for the estimation of sugar in small quantities of blood. *J. Biol. Chem.*, **20**:61-72.
108. MACLEOD, J. J. R.  
1921. The sugar of the blood. *Physiol. Rev.*, **1**:208-238.
109. MARRIOTT, W. M., AND J. HOWLAND  
1918. Phosphate retention as a factor in the production of acidosis in nephritis. *Arch. Int. Med.*, **18**:708-711.
110. MARSHALL, E. K. JR.  
1913. A new method for the determination of urea in blood. *J. Biol. Chem.*, **15**:487-494.
111. ——— AND D. M. DAVIS  
1914. Urea; distribution and elimination from the body. *J. Biol. Chem.*, **18**:53-80.
112. MEIGS, E. B., N. R. BLATHERWICK AND C. A. CARY  
1919. Contributions to the physiology of phosphorus and calcium as related to milk secretion. *J. Biol. Chem.*, **37**:1-73.
113. MOORHEAD, J. J., H. W. SCHMITZ, L. CUTTER AND V. C. MYERS  
1923. The phosphorus and calcium concentration of the serum of patients during the period of fracture union. *Proc. Am. Soc. Biol. Chem.*, xiii. *J. Biol. Chem.*, **55**.
114. MORGULIS, S., AND A. C. EDWARDS  
1924. Chemical changes in the blood during fasting and subsequent re-feeding experiments on dogs. *Am. J. Physiol.*, **68**:477-498.
115. MOSENTHAL, H. O., AND A. HILLER  
1919. Urea ntirogen and non-protein nitrogen of blood; relation between them. *J. Urol.*, **1**:75-89.
116. MYERS, V. C.  
1924. Chemical changes in the blood and their clinical significance. *Physiol. Rev.*, **4**:274-328.

117. \_\_\_\_\_  
1910. The physiology and pathology of creatinine and creatine. *Am. J. Med. Sci.*, **N. S.** 139:256-264.
118. \_\_\_\_\_  
1924. Practical chemical analysis of the blood. Mosby. Saint Louis, pp. 16-17.
119. \_\_\_\_\_ AND C. V. BAILEY  
1916. The Lewis and Benedict method for the estimation of blood sugar with some observations obtained from disease. *J. Biol. Chem.*, **24**:147-161.
120. \_\_\_\_\_, M. S. FINE AND W. G. LOUGH  
1916. The significance of the uric acid, urea and creatinine of the blood in nephritis. *Arch. Int. Med.*, **17**:570-583.
121. NEUMANN, A.  
1902. Einfache Veraschungsmethode (säuregemisch-Veraschung) und Vereinfachte Bestimmungen von Eisen, Phosphorsäure, Salzsäure und anderen Aschenbestandtheilen unter Benutzung dieser Säuregemisch-Veraschung. *Ztschr. Physiol. Chem.*, **37**:115-142.
122. NEWTON, E. B., AND A. R. DAVIS  
1922. Combined uric acid in human, horse, sheep, pig, dog and chicken blood. *J. Biol. Chem.*, **54**:603-605.
123. \_\_\_\_\_ AND \_\_\_\_\_  
1922. The distribution of the combined uric acid in the corpuscles of beef blood. *J. Biol. Chem.*, **54**:601-602.
124. PALMER, L. S., W. S. CUNNINGHAM AND C. H. ECKLES  
1930. Normal variations in the inorganic phosphorus of the blood of dairy cattle. *J. Dairy Sci.*, **13**:174-195.
125. PEARCE, R. J.  
1915. A criticism of the Bang and the Lewis-Benedict methods for the estimation of blood sugar with suggestions for a modification of the latter method. *J. Biol. Chem.*, **22**:525-533.
126. PETERS, J. P., AND L. EISERON  
1929. Influence of protein and inorganic phosphorus on serum calcium. *J. Biol. Chem.*, **84**:155-166.
127. POYALES, F.  
1927. Indice de creatinina en sangre; su valor pronostico en las lesiones retinianas de origine renal. *Progresos de la clinica*, **35**:20-22.
128. PREVOST, J. C., AND J. B. DUMAS  
1821. Examen du sang et de son action dans les divers phénomènes de la vie. *Bibliothèque universelle des sciences et des arts*, **18**:208-220.
129. PUCHER, G. W.  
1928. A study of the changes occurring in the blood and urine of puppies deprived of food and water. *J. Biol. Chem.*, **76**:319-329.
130. RICHTER, A.  
1928. Über die Tagesschwankungen des Blutzuckers beim Rinds. *Biochem. Ztschr.*, **194**:376-384.
131. ROBINSON, C. S., AND C. F. HUFFMAN  
1926. Studies on the chemical composition of beef blood. I. The concentration of certain constituents in normal beef plasma. *J. Biol. Chem.*, **67**:245-255.
132. RODERICK, L. M., AND A. F. SCHALK  
1927. Collected studies on hog cholera. *No. Dak. Ag. Exp. Sta. Bull.* 210.

133. ROE, J. H., AND O. J. IRISH  
1926. An accurate method for estimation of urea in blood and urine by direct nesslerization. *J. Lab. and Clin. Med.*, **11**:1087-1090.
134. RONA, P., AND D. TAKAHASHI  
1913. Beiträge zur Frage nach dem Verhalten des Calciums in Serum. *Biochem. Ztschr.*, **49**:370-380.
135. RUGGERI, G.  
1927. Comportaments del tasso glicemico in coniglie castrate, in seguito ad iniezione di diurentina. *Boll. Soc. Ital. di Biol. Sper.*, **2**:277-279.
136. SCHEUNERT, A., AND M. BARTSCH  
1923. Notiz über den Einfluss normaler Zugarbeit auf die Blutzusammensetzung des Pferdes. *Biochem. Ztschr.*, **139**:34-37.
137. ———— AND H. V. PELCHERZIM  
1923. Über den Gehalt des Blutes verschiedener Tierarten an Zucker, Rest-N., Harnstoffe-N., Kreatinenkörpern und Harnsäure nach den Folin'schen Methode. *Biochem. Ztschr.*, **139**:17-29.
138. SCHWARZ, K.  
1928. Untersuchungen über den normalen Blutzuckergehalt des Pferdes und des Rindes. *Biochem. Ztschr.*, **194**:328-334.
139. ———— AND H. HAMP  
1928. Über den normalen Blutzuckergehalt beim Hunde und seine physiologischen Schwankungen. *Biochem. Ztschr.*, **194**:351-361.
140. SEDGWICK, J. P.  
1910. Creatinine and creatine metabolism in children. *J. Am. Med. Assoc.*, **55**:1178-1180.
- 140a. SEVRINGHAUS, E. L., AND F. HIPPLE  
1925. Sources of error in blood urea and nitrogen determination. *J. Lab. and Clin. Med.*, **10**:934-935.
141. SHAFER, P. A.  
1908. The excretion of Kreatinin and Kreatin in health and disease. *Am. J. Physiol.*, **23**:1-22.
142. SHOPE, R. E.  
1928. The distribution of sugar between blood corpuscles and blood plasma for several animal species. *J. Biol. Chem.*, **78**:107-110.
143. TATUM, A. L.  
1920. Alkaline reserve capacity of whole blood and carbohydrate mobilization as affected by hemorrhage. *J. Biol. Chem.*, **41**:59-73.
144. TAYLOR, A. E., AND C. W. MILLER  
1914. On the estimation of phosphorus in biological material. *J. Biol. Chem.*, **18**:215-224.
145. TEICH, B.  
1928. Determinations de quelques constantes chimiques dans le sang des cobayes normaux. *Compt. rend. Soc. de Biol.*, **102**:151-153.
146. TISDALL, F. F.  
1923. A note on the Kramer-Tisdall method for the determination of calcium in small amounts of serum. *J. Biol. Chem.*, **56**:439-441.
147. ———— AND R. I. HARRIS  
1922. Calcium and phosphorus metabolism in patients with fractures. *J. Am. Med. Assoc.*, **79**:884-887.



148. TOLSTOI, E.  
1923. The inorganic phosphorus of the serum and plasma of 91 normal adults as determined by the Bell and Doisy method. *J. Biol. Chem.*, **55**:157-160.
149. VOEGTLIN, C., E. R. DUNN AND J. W. THOMPSON  
1924. The biological standardization of insulin. II. The mortality and glucose-protective test in rats and a method for the bio-assay of insulin. *Pub. Health. Rep. U. S. P. H. S.*, **39**:1935-1957.
150. WANG, C. C., AND M. L. DENTLER  
1920. Creatine and creatinine in blood. *J. Biol. Chem.*, **45**:237-243.
151. WAKEMAN, A. M., AND C. A. MORRELL  
1930. Chemistry and metabolism in experimental yellow fever in *Macacus rhesus* monkeys. I. Concentration of non-protein nitrogenous constituents in the blood. *Arch. Int. Med.*, **46**:290-305.
152. ——— AND ———  
1930. Chemistry and metabolism in experimental yellow fever in *Macacus rhesus* monkeys. II. Nitrogen metabolism. *Arch. Int. Med.*, **46**:382-401.
153. WATKINS, O., AND G. VAN S. SMITH  
1931. Biochemical studies on the effect of adrenalin upon the nitrogen metabolism of rabbits. *Am. J. Physiol.*, **96**:28-34.
154. WELLS, H. G.  
1918. The purine metabolism of the Dalmation coach hound. *J. Biol. Chem.*, **35**:221-225.
155. WILLS, L.  
1925. Blood calcium and inorganic phosphates in children with marked lack of muscle tone. *Brit. Med. J.*, **1**:302-304.
156. YOUNGBERG, G. E.  
1922. The use of open delivery tubes in distillation when determining urea and non-protein nitrogen. *J. Lab. and Clin. Med.*, **7**:552-555.

## LITERATURE EXAMINED BUT NOT CITED

- ABUREL, E., AND J. ORNSTEIN  
1930. La calcémie et leopoids des Nouveau-nés. *Compt. rend. Soc. de biol.*, **104**:1247-1249.
- ACKERSON, C. W., M. J. BLISH AND F. E. MUSSEHL  
1925. A study of the phosphorus calcium and alkaline reserve of the blood sera of normal and rachitic chicks. *J. Biol. Chem.*, **63**:75-84.
- ACKROYD, H.  
1910. Uric acid metabolism in dogs. *Biochem. J.*, **5**:217-224.
- ALLARDYCE, J., R. H. FLEMING, F. L. FOWLER AND R. H. CLARK  
1930. Blood normals for cattle: Some pathological values. *Canadian J. Research*, **3**:120-124.
- AMENDT, K.  
1922. Das Blut der Haustiere mit neuen Methoden untersucht. IV. Die Gerinnungszeit des Blutes der Haustiere. *Pflügers Arch. Physiol.*, **197**:556-567.
- ARON, H.  
1907. Eine einfache Methode zur Bestimmung des Calciums in Organischen Substanzen. *Biochem. Ztschr.* **4**:268-270.
- AUTENRIETH, W., AND FUNK, A.  
1914. Ueber Kolorimetrische Bestimmungsmethoden: Die Bestimmung der Harnsäure im Blut und Harn. *München Med. Wehnschr.*, **61**:457-461.

## BARRON, M.

1923. The value and importance of blood chemistry in clinical medicine. *Minn. Med.*, **6**:238-244.

## BENEDICT, F. G., AND V. C. MYERS

1907. The determination of creatine and creatinine. *Am. J. Physiol.*, **18**:397-405.

\_\_\_\_\_, S. R.

1925. The determination of uric acid in the blood. *J. Biol. Chem.*, **64**:215-219.

\_\_\_\_\_, E. OSTERBERG AND I. NEUWIRTH

1918. Studies in carbohydrate metabolism. II. A study of urinary sugar excretion in two normal men. *J. Biol. Chem.*, **34**:217-262.

## BERGLUND, H.

1922. Nitrogen retention in chronic interstitial nephritis and its significance. *J. Am. Med. Assoc.*, **79**:1375-1380.

## BIGELOW, E. B.

1921. Review of 300 cases of blood chemistry. *Boston M. and S. J.*, **184**:459.

## BIGWOOD, E. J.

1930. L'analyse chimique du sang peut-elle apporter un renseignement utile au médecin légiste dans le diagnostic post-mortem de la mort par coma diabétique ou néphritique? *Ann. de med. leg.*, **10**:284-296.

## BLOUNT, W. P.

1930. Haematology in veterinary practice. *Vet. Rec. n.s.* **10**:207-209.

## BREUER, M. J.

1925. Blood nitrogen method for the practitioner. *Neb. State Med. J.*, **10**:178-179.

## BÜRGER, U.

1928. Das Blutbild moribunder Pferde. *Arch. f. Wissensch. u. prakt. Tierh.*, **58**:54-67.

## BURIAN, R.

1905. Über die oxydative und die vermeintliche synthetische Bildung von Harnsäure in Rinderleberauszug. *Ztschr. f. physio. chem.*, **43**:497-531.

## BYRD, T. S.

1930. Blood chemistry and internal medicine; aid in diagnosis, prognosis and treatment. *J. M. A. Georgia*, **19**:420-424.

## CLARK, E. P., AND J. B. COLLIP

1926. A procedure for the determination of urea in Folin-Wu blood filtrates by the autoclave method. *J. Biol. Chem.*, **67**:621-627.

## CLOSSON,, O. E.

1906. The elimination of creatinin. *Am. J. Physiol.*, **16**:252-267.

## CHAPMAN, A. C.

1909. On Jaffé's colorimetric method for the estimation of creatinine. *Analyst*, **34**:475-483, London.

## CHEYMOL, J., AND A. QUINQUAND

1930. Constance du taux du calcium sanguin chez la lapin, devant l'infection de substances paralysante du sympathique (yohimbine, ergotamina, bleu de méthylène), *Arch. internat. de Pharmacodyn et de thérapie*, **38**:431-433.

## COLLIP, J. B.

1927. Composition of the blood and tissues of the foetal calf. *Trans. Roy. Soc. Canada (Sect. V. Biol. Sc.)*, **21**:147-150.

## COOK, J. V.

1910. The excretion of calcium and magnesium after parathyroidectomy. *J. Exp. Med.*, **12**:45-58.

- 
- \_\_\_\_\_, F. H. RODENBAUGH AND G. H. WHIPPLE  
1916. Intestinal obstruction. VI. A study of non-coagulable nitrogen of the blood. *J. Exp. Med.*, **23**:717-738.
- CRISTOL, P. A., A. PUECH AND P. MONNIER  
1929. Repartition de l'azote non-proteic azote dans les globules et le plasma. Variations en fonction de l'azotémie. *Arch. Soc. d. Sc. Med. et biol. de Montpellier*, **10**:200-203.
- CSONKA, F. A., AND G. C. TAGGART  
1922. Note on the reliability of the Benedict and Folin-Wu blood sugar determinations. *J. Biol. Chem.*, **54**:1-3.
- DENIS, W., AND J. L. BEVEN  
1923-24. Methods of preservation of specimens of blood intended for the determination of the non-protein organic constituents. *J. Lab. and Clin. Med.*, **9**:674-679.
- 
- \_\_\_\_\_, AND J. GODDARD  
1923. A study of inorganic constituents of the blood in experimental nephritis. *J. Biol. Chem.*, **56**:473-481.
- DIEZ, FERNANDEZ C.  
1930. Contribution al estudio de la azoemia en enfermedades infecciosas. *Rev. espan. de Med. y cir.*, **13**:260-263.
- DIONNE, M. J., AND J. J. ARENSTAM  
1930. The fluctuations of the capillary blood sugar in normal young women during a twenty-four hour period. *J. Biol. Chem.*, **87**:393-397.
- DOLHAINE, H.  
1926. Beitrag zur Frage der in Serum vorkommenden Calciumphosphatverbindungen. *Biochem. Ztschr.*, **178**:233-242.
- DORNER, G.  
1907. Zur Bildung von Kreatin und Kreatinin im Organismus besonders der Kaninchen. *Ztschr. f. physiol.*, **52**:225-278.
- DUPRAY, M.  
1927. A modified digestion acid for non-protein nitrogen determination. *J. Lab. and Clin. Med.* **12**:387.
- DUVAL, C. W.  
1929. Observations upon the nature of the virus of hog cholera. *Proc. Soc. Exp. Biol. and Med.*, **27**:87-89.
- ELIAS, H., AND FELL, B.  
1930. Zur Physiologie und Pharmakologie des Kritischen Blutzuckers. *Arch. f. exper. Path. u. Pharmakol.*, **150**:146-159.
- EVERETT, M. R., H. A. SHOEMAKER AND F. SHEPPARD  
1927. Total sugar of blood and urine. *J. Biol. Chem.*, **74**:739-759.
- FALISI, J. V., AND V. A. LAWTON  
1924. Tables for blood chemistry calculations. *J. Lab. and Clin. Med.*, **9**:566-571.
- FIESSINGER, N.  
1929. Les azotémies sans signification diagnostique ni pronostique. (*Rev. gén. de chin. et de therap.*), **43**:593-598.
- FISKE, C. H., AND J. B. SUMNER  
1914. The importance of the liver in urea formation from amino acids. *J. Bio. Chem.*, **18**:285-295.
- FOLIN, O.  
1908. Chemical problems in hospital practice. *J. Am. Med. Assoc.*, **50**:1391-1394.

- 
1904. Beitrag zur chemie des Kreatinins und Kreatins im Harne. Ztschr. f. phys. chem., **41**:223-242.
- 
1915. Note in defense of the Folin-Farmer method for the determination of nitrogen. J. Biol. Chem., **21**:195-199.
- 
1907. On the occurrence and formation of alkyl ureas and alkyl amines. J. Biol. Chem., **3**:83-86.
- 
- AND A. SVEDBERG
1930. Diffusible non-protein constituents of blood and their distribution between plasma and corpuseles. J. Biol. Chem., **88**:715-728.
- 
- AND —
1930. Micro-methods for the determination of non-protein nitrogen, urea, uric acid and sugar in unlaked blood. J. Biol. Chem., **88**:85-96.
- 
- , H. C. TRIMBLE AND L. H. NEWMANN
1927. The distribution and recovery of glucose injected into animals. J. Biol. Chem., **75**:263-281.
- FORBES, E. B., J. HALVERSON AND J. A. SCHULZ
1920. Alkali reserve of swine as affected by cereal feeding and mineral supplements. J. Biol. Chem., **42**:459-463.
- FORD, B. C.
1923. Value of blood chemistry in clinical diagnosis. Wisc. Med. J., **21**:397-400.
- FOSTER, N. B., AND H. S. FISHER
1911. Creatin and creatinin metabolism in dogs with eck fistula. J. Biol. Chem., **9**:359-362.
- 
- AND J. C. GREENWAY
- 1908-9. Synthesis of uric acid. Proc. Soc. Exp. Biol. and Med., **6**:76-77.
- FREI, W., AND M. A. EMMERSON
1930. Der Serumkalkspiegel beim Rinde mit Besonderer Berücksichtigung der Beziehungen zum Geschlechtsapparat. Biochem. Ztschr., **226**:355-380.
- FRITSCH, G.
1920. Das Blut der Haustiere mit neuen Methoden Untersucht. II. Untersuchung des Kaninchen,—Hühner—und Tauben—blutes. Pflügers Arch. f. Physiol., **181**:78-105.
- FRONTZ, W. A., AND J. I. GERAGHTY
1922. The value of the estimation of urea, non-protein nitrogen and creatinine as an index of renal function. J. Am. Med. Assn., **79**:1383-1384.
- GAEBLER, O. H.
1930. Further studies of blood creatinine. J. Biol. Chem., **89**:451-466.
- GAVERILIA, I., V. VIORE AND RAMNEANTZU
1929. La créatininémie et la créatinémie dans quelques états pathologiques. Compt. rend. Soc. de biol., **100**:381-383.
- GERLI, P.
1930. Dell ' istamina con particolare riguardo alle modificazioni biochemiche e morfologiche che essa induce nel sangue. Osp. Magg., **17**:235-249, 1929 (original not seen). Abstract—Berichte über die Gesamte Physiologie, **53**:143.
- GREENWALD, I.
1925. A new type of phosphoric acid compound isolated from blood with some remarks on the effect of substitution on the rotation of l-glyceric acid. J. Biol. Chem., **63**:339-349.



- GREISHEIMER, E. M., O. H. JOHNSON AND M. RYAN  
1929. Relationship between serum calcium and age. *Am. J. Med. Sc.*, 177:704-710.
- GROLLMAN, A.  
1927. The condition of inorganic phosphorus of the blood with special reference to calcium concentration. *J. Biol. Chem.*, 72:565-572.
- GUILLAUMIN, C. O., AND H. VIGNES  
1928. Composition du sang et cycle menstruel. Recherche du sucre et du phosphore. *Comp. rend. Soc. de biol.*, 99:749-752.
- HARDING, V. C.  
1929. Note on some urine and blood chemistry in milk fever. *J. Am. Vet. Med. Assn.*, 75:702-704.
- \_\_\_\_\_, H. MURPHY AND C. E. DOWN  
1928. Observations on blood sugar and serum calcium in relation to lactation in women, with a study of its possible relationship to parturient paresis. *Am. J. Obst. and Gynec.*, 16:765-783.
- HAYDEN, C. E.  
1930. The constituents of the blood of animals as evidence of intestinal contribution to the cause of diseases of obscure origin. *Cornell Vet.*, 20:223-231.
- HERREL, H.  
1922. Das Blut der Haustiere mit neueren Methoden Untersucht III. Differentialzählungen der Lymphocyten und Monocyten in Pferde—, Rinder—, und Hundeblut. *Pflügers Arch. f. Physiol.*, 196:560-570.
- HILL, L. W.  
1923. A review of the literature on the blood and blood diseases of infants and children for the last four years. (1918-1921.) *Am. J. Dis. Chil.*, 25:168-184.
- HOLBROOK, W. P., AND H. D. HOSKINS  
1926. Blood uric acid in nephritis. *J. Lab. and Clin. Med.*, 12:10-15.
- HOPKINS, A. H.  
1915. Studies in the concentration of blood sugar in health and disease as determined by Bang's micro-method. *Am. J. Med. Sc.*, 149:254-267.
- HORVATH, A. A.  
1928. The effect of yeast feeding on some blood constituents of hens. *Am. J. Physiol.*, 87:208-220.
- HOWE, P. E.  
1925. The function of plasma proteins. *Physiol. Rev.*, 5:439-476.
- HOWLAND, J., AND W. MCK. MARRIOTT  
1917-18. Observations upon the calcium content of the blood in infantile tetany and upon the effect of treatment by calcium. *Quart. J. Med.*, 11:289-319.
- HUBBARD, R. S.  
1923. Creatinine determinations in the blood. *Clifton Med. Bul.*, 10:10-12.
- \_\_\_\_\_  
1928. Some laboratory findings in a case of mesenteric vascular thrombosis. *Clifton Med. Bul.*, 14:90-95.
- HUGHES, T. A., D. L. SHRIVASTAVA AND P. N. SAHAI  
1929. Observations on serum calcium and inorganic phosphorus in health and disease. *Indian J. Med. Res.*, 17:461-469.
- HUNTER, G., AND B. A. EAGLES  
1925. The isolation from blood of a hitherto unknown substance, and its bearing on present methods for the estimation of uric acid. *J. Biol. Chem.*, 65:623-641.
- IRVING, L., AND G. M. BASTEDO  
1928. The inorganic phosphorus in blood and muscle. *Am. J. Physiol.*, 86:225-237.

ISVEKOV, V. G.

1930. Analysis of blood and functional tests as aid in diagnosis. *J. Okla. Med. Assn.*, **23**:310-316.

IZAR, G.

1910. Beiträge zur Kenntnis der Harnsäure Bildung. V. Mitteilung. *Ztschr. f. physiol. Chem.*, **64**:62-66.

---

1910. Beiträge zur Kenntnis der Harnsäure Bildung. VI. Mitteilung. *Ztschr. f. physiol. Chem.*, **65**:78-88.

JOHNSON, R. L.

1930. A study of the blood-urea clearances with relation to diuresis in normal and nephritic animals. *J. Lab. and Clin. Med.*, **15**:943-952.

AF KLERCKER, K. O.

1906. Zur Frage der Kreatin- und der Kreatininausscheidung beim Menschen. *Beitr. z. chem. Physiol. u. Path.*, **8**:59-61.

---

1907. Beitrag zur Kenntnis des Kreatins and Kreatinins in Stoffwechsel des Menschen. *Biochem. Ztschr.*, **3**:45-87.

KUHL, P.

1919. Das Blut der Haustiere mit neuen Methoden untersucht. I. Untersuchung des Pferde-, Rinder- und Hundblutes. *Pflügers Arch. f. Physiol.*, **176**:263-284.

LASCH, G., AND J. REITSTÖTTER

1928. Berichtigung zu unserer Arbeit: "Zu physikalisch-chemischen Kennzeichnung von normalem und pathologisch Verändertem Blutserum" und Bemerkungen zu: "Elektrodialyse oder Elektrosmose" von L. Reiner. *Biochem. Ztschr.*, **199**:216-217.

LÉPINE, R., AND BOULUD

1906. Sur la nature du sucre virtuel du sang. *Compt. rend. Acad. d. Sc.*, **143**:500-504.

LEVINE, P. A., AND L. KRISTELLER

1909. Factors regulating the creatinin output in man. *Am. J. Physiol.*, **24**:45-65.

LEWIS, R. C., AND S. R. BENEDICT

1913-14. A method for the estimation of sugar in small quantities of blood. *Proc. Soc. Exper. Biol. and Med.*, **11**:57-58.

LIU, R. K. S., AND T. G. NI

1924-26. Changes in blood constituents accompanying gastric secretion. II. Blood volume (hemoglobin oxygen capacity, relative volume and total solids). *Chinese J. Physiol.*, **1**:199-211, 1927. *Am. J. Physiol.*, **75**:475-486.

LITTLE, W. L., T. S. KEITH AND H. T. FAWNS

1928. The blood sugar of the cow in milk fever and under normal conditions. *Vet. J.*, **84**:284-287.

LUY, P.

1928. Zur Kryoskopie des normalen Pferdeblutes. *Deutsche tierärztl. Wchnschr. (Sondernum)*, **36**:32-38.

MACCOLLUM, W. G., AND C. VOEGTLIN

1909. On the relation of tetany to the parathyroid glands and to calcium metabolism. *J. Exper. Med.*, **11**:118-151.

MACHEBOEUF.

1927. Recherches sur le phosphore du serum. *Ann. de l' inst. Pasteur*, **41**:1026-1044.

MACLEOD, J. J. R.

1908. A brief survey of the development of physiological knowledge from the time of the Reformation to the beginning of the nineteenth century. *Cleveland Md. J.*, **7**:121-135.

McMASTER, P. D., AND D. R. DEURY

1929. The production of partial liver insufficiency in rabbits. *J. Exp. Med.*, **49**:745-758.

MAGEE, H. E., AND D. HARVEY

1927. The effect of insulin on the blood sugar of pigs. *Proc. Physiol. Soc.* 1927. *J. Physiol.*, **64**:xxxi-xxxii.

MANN, F. C.

1925. Modified physiologic processes following total removal of the liver. *J. Am. Med. Assn.*, **85**:1472-1475.

MARIE, A.

1930. Recherches sur l'urée dans le sang des animaux. *Ann. de l'inst. Pasteur*, **36**:820-829.

MARINO, S.

1927. Testicoli e ricambio degli idrati di carbonio. Influenza dei testicoli sulla reazione glicemica alimentare ed all'adrenalina. *Policlinico (Sezione Medica)*, **34**:301-308.

MARSCHAK, M., AND O. DUKESEY

1929. Untersuchungen über die Wärmeregulation. II. Mitteilung. Über die Wirkung hoher Umgebungstemperaturen auf den physikalischen Zustand des Blutes und auf die Wärmeregulation bei Menschen in Verbindung mit Wasser- und Kochsalzaufnahme. *Arch. f. Hyg.*, **101**:325-337.

MELLANBY, E.

1907-8. Creatin and creatinin. *J. Physiol.*, **36**:447-487.

MAYO, W. J.

1928. Advances in surgery thru physiochemic studies of blood. *J. Iowa Med. Soc.*, **15**:105-108.

MENDEL, L. B.

1906. The formation of uric acid. The Harvey lectures, p. 195-218, Lippincott, Philadelphia. 1905-06. *J. Am. Med. Assn.*, **46**:843-846.

---

1909. The physiological significance of creatin and creatinin. *Science*, n.s., **29**:584-591.

---

— AND O. E. CLOSSON

1904-05. On the elimination of creatinine. *Proc. Am. Physiol. Soc.*, *Am. J. Physiol.*, **13**:xix.

---

— AND J. F. LYMAN

1910-11. The metabolism of some purine compounds in the rabbit, dog, pig and man. *J. Biol. Chem.*, **8**:115-143.

MIRKIN, A., AND S. J. DRUSKIN

1923. A new method for the determination of calcium, magnesium, potassium and sodium in human blood. *J. Lab. and Clin. Med.*, **8**:334-339.

MOREAU, E., AND J. DIAMANT

1923. Diagnosis and prognosis of uremic nephritis by means of clinical dosage of creatinin in blood. *Progrès Méd.*, **38**:341-342.

MOSENTHAL, H. O., S. W. CLAUSEN AND A. HILLER

1918. The effect of diet on blood sugar in diabetes mellitus. *Arch. Int. Med.*, **21**:93-108.

MUNTUYLER, E., G. B. RAY, V. C. MYERS AND T. SOLLMAN

1929. Blood changes in victims of the Cleveland Clinic fire disaster. *J. Am. Med. Assn.*, **93**:512-513.

MYERS, V. C., AND M. S. FINE

1914. Studies on the relationship between creatine and creatinine. *Proc. Soc. Exp. Biol. and Med.*, **12**:41-42.

---

— AND J. A. KILLIAN

1919. The prognostic value of the creatinine of the blood in nephritis. *Am. J. Med. Sc.*, **157**:674-695.

---

— AND W. G. LOUGH

1915. The creatinine content of the blood in nephritis. Its diagnostic value. *Arch. Int. Med.*, **16**:536-546.

NEAL, W. M., L. S. PALMER, C. H. ECKLES AND T. W. GULLICKSON

1931. The effect of age and nutrition on the calcium phosphate / calcium carbonate ratio in the bones of cattle. *J. Agr. Res.*, **42**:115-121.

NORRIS, J. H.

1931. A chemical investigation in Victoria (Australia) of the blood of cattle and sheep. *Australia J. Exp. Biol. and Med.*, **7**:3-4, 1930. (Abstr.) *Physiol. Abstr.*, **16**:21.

OPPENHEIMER, C.

1907. Über die Frage der Anteilnahme elementaren Stickstoffs am Stoffwechsel der Tiere. Eine historisch-Kritische und experimentelle Studie. *Biochem. Ztschr.*, **4**:328-470.

PATCH, F. S., AND I. M. RABINOWITCH

1928. Urea and creatinine content of the blood in renal disease; statistical analysis of 5,000 observations. *J. Am. Med. Assn.*, **90**:1092-1095.

PATTERSON, S. W.

1908. A contribution to the study of calcium metabolism. *Biochem. J.*, **3**:39-54.

PAYNE, W. W.

1924. Hagedorn-Jensen method of estimating blood sugar. *Guy's Hosp. Rep.*, **74**:240-241.

PETRÉN, K., AND M. ODIN

1925. Valeur de l'azote non proteique total dans le coma diabétique et hypoglycémique. *Compt. rend. Soc. de biol.*, **93**:375-376.

PEYRE, E.

1928. Quantités comparées d'albumines totales et d'urée dans le sérum sanguin. *Compt. rend. Soc. de biol.*, **98**:96-98.

PICKARD, R. J., AND L. F. PIERCE

1930. Blood dextrose determinations. A statistical comparison of the Folin-Wu method and the Benedict modification of the Lewis-Benedict method. *J. Am. Med. Assn.*, **94**:480-483.

POLAYES, S. H., E. HERSHEY AND M. LEDERER

1930. Post mortem blood chemistry in renal disease. *Arch. Int. Med.*, **46**:283-289.

RANDLE, F. S., AND W. K. GRIGG

1924. Estimation of blood sugar by the Folin-Wu method using one-tenth cubic centimeter of blood. *J. Am. Med. Assn.*, **82**:684-686.

RÉMOND AND ROUZAUD

1923. I. Valeurs comparées du glucose et de l'acide urique dans le plasma sanguin. *Bul. Acad. de Med.*, **89**:585-586, 1923. (Abstr.) *J. Am. Med. Assn.*, **81**:863.

ROBINSON, C. S., AND C. F. HUFFMAN

1926. Studies on the chemical composition of beef blood. II. The composition of blood of dams and calves immediately after calving. *J. Biol. Chem.*, **67**:257-266.



- ROCKWOOD, R.  
1928. Chemical tests of blood. Indications and interpretations. *J. Am. Med. Assn.*, **91**:157-166.
- RONDONI, P.  
1928. Significato patogenetico dell' uricemia. *Riforma Med.*, **44**:771-776.
- RUDOLF, J.  
1928. Hämatologische Studien bei einigen Krankheiten des Rindes des Schweines und der Ziege. *Deutsche tierärztl. Wehnschr.*, **36**:445-451.
- SCHERK, G.  
1929. Harnsäurestudien an Blut und Gewebssaft. *Ztschr. f. Klin. Med.*, **111**:167-178.
- SCHMIDT, J.  
1928. Toxische Hyperproteinämie als Todesursache bei Pferden und Schaafen. *Berl. Tierärztl. Wehnschr.*, **44**:309-310.
- SCHWARZ, K., AND K. HEINRICH  
1928. Untersuchungen über den normalen Blutzuckergehalt des Huhnes. *Biochem. Ztschr.*, **194**:346-350.
- SEEMAN, J.  
1907. Beitrag zur Frage der Kreatininbildung. *Ztschr. f. Biol.*, **49**:333-344.
- SHAFFER, P.  
1908. The excretion of kreatinin and kreatin in health and disease. *Am. J. Physiol.*, **23**:1-22.
- SHERMAN, H. C.  
1907. Experiments upon the total metabolism of iron and calcium in man. *Proc. Soc. Exp. Biol. and Med.*, **4**:21-22, 1906. *Science*, **25**:421.
- SHOPE, R. E.  
1927. Sugar and cholesterol in the blood serum as related to fasting. *J. Biol. Chem.*, **75**:101-113.
- SKEKANEK, G., AND K. OTTER  
1928. Untersuchungen über den Reststickstoffgehalt in Blut plasma des Pferdes und des Rindes. *Arch. f. Wissensch. u. prakt. Tierh.*, **57**:567-574.
- SMITH, C. S., AND A. L. BROWN  
1923. A quantitative method for the determination of total phosphorus in blood. *J. Lab. and Clin. Med.*, **9**:203-205.
- SQUIER, J. B., C. G. BANDLER AND V. C. MYERS  
1922. Significance of chemical blood findings in urologic conditions. *J. Am. Med. Assn.*, **79**:1384-1386.
- TALBERT, G. A., S. SILVERS AND W. JOHNSON  
1927. Simultaneous study of constituents of the sweat, urine and blood, also gastric acidity and other manifestations resulting from sweating; II. Total nitrogen of sweat and urine; total non-protein nitrogen of blood. *Am. J. Physiol.*, **81**:81-85.
- TAYLER, A. E.  
1911. The source of error in the Folin method for the estimation of creatinine. *J. Biol. Chem.*, **9**:19-20.
- TILESTON, W., AND C. W. COMFORT, JR.  
1914. The total non-protein nitrogen and urea of the blood in health and disease as estimated by Folin's methods. *Arch. Int. Med.*, **14**:620-649.
- TROWBRIDGE, P. F., AND L. STANLEY  
1910. Phosphorus in flesh. *J. Ind. and Eng. Chem.*, **2**:212-215.

UNDERHILL, S. W. F.

1923. The relative concentration ratios of some constituents of urine. I. The distribution between plasma and corpuscles, and condition in blood, of urea, creatinine, inorganic phosphate and uric acid. *Brit. J. Exp. Path.*, **4**:87-91.

VAN HOOGENHUYZE, C. J. C., AND H. VEGPLOEGH

1905. Beobachtungen über die Kreatininausscheidung beim Menschen. *Ztschr. f. physiol. chem.*, **48**:415-471.

VAN SLYKE, D. D., AND G. E. CULLEN

1914. A permanent preparation of urease, and its use for rapid and accurate determination of urea. *J. Am. Med. Assn.*, **62**:1558-1559.

VOLKER, R.

1929. Blutzuckeruntersuchungen an gesunden und kranken Tieren. II. Mitteilung. *Arch. f. Wissensch. u. prakt. Tierh.*, **60**:467-506.

WANG, C. C., AND A. R. FELSHER

1925. The effect of hemolysis on the calcium and inorganic phosphorus content of serum and plasma. *J. Lab. and Clin. Med.*, **10**:269-272.

WAY, C. T.

1926. Practical blood chemistry. *Ohio State Med. J.*, **22**:499-500.

WHITTIER, A. C.

1911. Investigations on the estimation of inorganic phosphorus in animal tissues. *J. Ind. and Eng. Chem.*, **3**:248-250.

# CERTAIN CHEMICAL AND MORPHOLOGIC PHASES OF THE BLOOD OF NORMAL AND CHOLERA-INFECTED SWINE

## II. CERTAIN MORPHOLOGIC PHASES

W. T. OGLESBY, E. A. HEWITT AND H. D. BERGMAN

*From the Department of Veterinary Physiology and Pharmacology, Iowa State College*

Accepted for publication December 15, 1931

This problem was undertaken with the object of determining whether an examination of the morphologic blood picture would be of value in the diagnosis of hog cholera. At present the most reliable laboratory method of making a positive diagnosis of hog cholera is by the inoculation of a susceptible pig with the suspect's blood, which has been passed through a Berkefeld filter. In the field a diagnosis is made from the history, symptoms and general gross post-mortem lesions.

The outstanding gross lesions of acute uncomplicated hog cholera are petechia or ecchymoses of the kidneys, lungs and, less frequently, of the liver. These lesions are found also in the upper respiratory passages, the bladder and the stomach. All lymph nodes are markedly congested, those of the mesenteric, cervical and submaxillary regions being most commonly observed. Not uncommonly a diseased animal dies from the peracute form of cholera with no apparent gross lesions manifested. In the acute, sub-acute or chronic forms the primary lesions mentioned may be largely complicated by lesions due to secondary infection.

Few detailed studies have been made of the blood in swine diseases. In fact, there are relatively meager data available of either the chemistry or morphology of the blood of normal swine. Furthermore, there are no symptoms or gross pathologic lesions which may be regarded as strictly pathognomonic of hog cholera. In view of these facts and the extreme economic importance of successfully controlling hog cholera, the most widespread and fatal disease of swine, it was thought that comparisons of the morphologic characteristics of normal and cholera-infected blood of swine might prove of considerable significance.

It is recognized that morphologic alterations in the blood are of diagnostic importance in certain diseases. If such proved to be the case in hog cholera, a laboratory blood examination might prove an extremely valuable adjuvant in the definite diagnosis of this disease where the gross lesions are not of such a nature to enable a positive diagnosis.

## REVIEW OF LITERATURE

A review of the literature reveals few references concerning the morphologic phases of normal swine blood.

### BLOOD OF NORMAL SWINE

#### *Erythrocytes*

Bethe (158) found 6,960,000 erythrocytes per c.mm. of blood to be an average for a limited number of pigs. Storch (182) made observations on

sixteen pigs which showed the following values for erythrocytes per c.mm. of blood. Five castrated males nine months of age averaged 8,432,000, and 3 females, 9 months of age averaged 7,660,000. The average for the 8 pigs was 8,046,000. Four male pigs varying from 3 to 35 days of age averaged 5,140,000 and 4 female pigs varying in age from 6 to 28 days averaged 4,710,000. The average for these 8 pigs was 4,925,000, whereas the average for the group of 16 pigs was 6,485,000.

It is noted that the older pigs showed higher counts than the younger pigs. It is interesting that in both groups the male animals showed higher counts than the females.

Allumbaugh (157) reported that blood samples from 50 human infants showed an average of 1,310,000 more erythrocytes per c.mm. than did the blood of their mothers.

Giltner (162) found that the mean number of erythrocytes for 24 pigs, averaging four months of age, was 8,450,000 per c.mm. of blood. The counts ranged between 6,800,000 and 8,800,000 per c.mm. Regner (177) found 3,632,000 erythrocytes was the average in suckling pigs. He stated that the count increased rapidly after weaning.

Gütig (163) studied the effects of feeding and starvation on the cellular elements of swine blood. He found the erythrocyte counts in 13 normal pigs ranged from 2,900,000 to 7,080,000 per c.mm., with an average of 5,921,000. Palmer (176) found that the mean for 25 pigs, between the ages of 2 and 42 days, was 3,855,000 erythrocytes per c.mm. of blood; whereas, for 25 pigs weighing about 100 pounds the mean was 6,215,160 per c.mm. The counts were higher in the well nourished than in the poorly nourished pigs. Palmer (175) also studied the blood of 15 pigs and noted the effect of exercise on the number of erythrocytes. He found a mean count of 6,098,233 per c.mm., exercise having produced no significant change.

Lütje (170) found 8,250,000 erythrocytes per c.mm. to be the mean number in the blood of four pigs six weeks of age, and 8,590,000 in four older pigs. Senftleben (179) reported the erythrocyte counts in fifty normal swine ranged from 2,892,000 to 9,600,000. The mean for seventeen suckling pigs was 4,206,250, and 8,963,900 was the mean for 33 mature pigs. The mean for the entire group was 7,346,380 per c.mm. Senftleben found the average count was 7,200,000 at four months of age. This remained constant until about the fourth year, when it dropped to 6,300,000 per c.mm. He stated that the variations found were due to such factors as age, sex, types of feed, time of feeding and time of bleeding. He also found that the young males had a higher count than the young females, and he further stated that pigs were born with a very low erythrocyte count as compared to human infants.

Welsch (185) found the average number of erythrocytes in 5 male and 5 female pigs was 7,440,000 per c.mm., with the males 300,000 higher than the females. Naujek (174) reported 7,200,000 red corpuscles as the average number for swine. Hikmet (164) reported the erythrocyte count ranged from 6,280,000 to 7,736,000, with a mean of 7,256,800 per c.mm. in five animals.

King and Wilson (166) made erythrocyte counts on 43 normal pigs and found them to range from 4,000,000 to 9,360,000, with a mean of 6,458,000 per c.mm. of blood. Dinwiddie (160) observed 15 pigs and found that the red corpuscles varied between 2,090,000 and 9,000,000, with an average of



6,348,600 per c.mm. Lewis and Shope (169) found that the red corpuscle counts fluctuated between 6,500,000 and 7,500,000 per c.mm. in 8 normal pigs. In a later report the same authors (168) found that the erythrocytes in the blood of 6 pigs ranged from 6,569,000 to 7,220,000, with an average of 6,825,160 per c.mm.

Wetzel (186) and Marloff (171) reported erythrocyte counts that agreed closely with those presented by other investigators.

### *Hemoglobin*

References to the hemoglobin content of swine blood are meager.

Müller (173) made hemoglobin determinations, using a spectroscopic method. He found 13.71 grams of hemoglobin per 100 c.c. of blood in one pig three years of age, 13.52 grams in one pig four years of age, and 13.26 grams as the average for three pigs one year of age. Giltner (162), using the Tallquist's method, found the minimum hemoglobin content of swine blood was 80 per cent, the maximum 100 per cent and the average 88 per cent in 24 pigs. Palmer (176), using a Sahli hemoglobinometer, found that the average hemoglobin content was 79.4 per cent in older pigs as compared to 56.8 per cent in younger animals. There were 25 pigs in each group.

Welsch (185) found an average of 16.8 grams of homeglobin per 100 c.c. of blood in males and 15.4 grams in females. Regner (177) reported the hemoglobin content was 30 per cent in suckling pigs. This increased very rapidly after weaning, as did also the erythrocyte count. Senftleben (179), using a Sahli hemoglobinometer, reported an average of 53 per cent for 17 suckling pigs and 91 per cent for 33 mature pigs. The average for the group was 72 per cent. Hikmet (164) found the hemoglobin content averaged 92 per cent in five pigs.

Allumbaugh (157) found the blood of 50 human infants contained 5.95 grams more hemoglobin per 100 c.c. than did the blood of their mothers.

King and Wilson (166), using a Dare hemoglobinometer, found the hemoglobin varied between 78 and 100 per cent, with an average of 83.06 per cent. Lewis and co-workers (167) found the hemoglobin content was 80 and 90 per cent, respectively, in two pigs.

### *Leucocytes*

Bethe (158) gave 7,840 leucocytes per c.mm. of blood as the average for the blood of normal swine. Storch (182) reported an average of 11,490 leucocytes per c.mm. in six young pigs. Giltner (162) studied 24 normal animals and found wide variations in the total and differential leucocyte counts. His results are as follows: total leucocyte count showed a mean of 19,000, with a maximum of 25,000 and a minimum of 9,500. The differential counts gave a mean for lymphocytes of 51.6 per cent, with a maximum of 79.8 per cent and a minimum of 30 per cent, mononuclears averaged 4.6 per cent, with a maximum of 10 per cent and a minimum of 4.6 per cent, polymorphonuclears averaged 37 per cent, with a maximum of 60 per cent and a minimum of 13 per cent, eosinophils averaged 5.2 per cent, with a maximum of 11 per cent and a minimum of 5.2 per cent, mast cells averaged 1.3 per cent, with a maximum of 5.6 per cent and a minimum of 1.3 per cent.

There is a wide range in the total number of leucocytes. A significant feature is that the lymphocytes outnumber the polymorphonuclears.

Palmer (176) gave 18,320 leucocytes per c.mm. in older pigs, compared with 13,500 for younger pigs. Each group consisted of 25 pigs. The average for the two groups was 15,910. The older group showed 52.21 per cent lymphocytes, 39.79 per cent polymorphonuclears, 0.79 per cent mononuclears, 3.42 per cent eosinophils and 0.79 per cent basophils. The younger group showed 63.25 per cent lymphocytes, 32.14 per cent polymorphonuclears, 2.63 per cent mononuclears, 1.29 per cent eosinophils and 0.25 per cent basophils. The averages for the two groups were: lymphocytes 57.73 per cent, polymorphonuclears 33.98 per cent, mononuclears 1.71 per cent, eosinophils 2.35 per cent, and basophils 0.52 per cent.

Here again a significant fact is that the lymphocytes outnumber the polymorphonuclears. The lymphocytes are higher in the younger than in the older pigs. The polymorphonuclears are higher in the pigs of the older group.

Senftleben (179) made seventeen counts on suckling pigs and found the leucocytes ranged from 8,850 to, in one case, 40,400, with an average, however, of 13,900 per c.mm. The counts on 33 mature pigs ranged from 12,000 to 29,000, with an average of 20,200. The significant features of his observations are as follows: the lymphocytes averaged 55.92 per cent, with a maximum of 69.75 per cent and a minimum of 24 per cent. The mononuclears averaged 3.43 per cent, with a maximum of 6.25 per cent and a minimum of 1.25 per cent. The polymorphonuclears averaged 38.03 per cent, with a maximum of 92 per cent and a minimum of 25 per cent. The eosinophils averaged 1.89 per cent and varied between 0.0 and 8.25 per cent. The basophils averaged 0.93 per cent and varied between 0.0 and 2.25 per cent.

These results also showed that the lymphocytes constituted the majority of the total leucocytes. Senftleben stated that there was little essential difference in the morphology of the leucocytes of human, swine and horse bloods. He also stated that it was often difficult to differentiate between the lymphocytes and mononuclears in blood from animals of the same species.

Regner (177) reported that the total and differential leucocyte counts varied greatly in periods of feeding and starvation, and that sex and age were also responsible for variations.

Welsch (185) found the average leucocyte count of 5 male and 5 female pigs was 17,120 per c.mm. The count was slightly higher in the males than in the females. A differential count on these pigs revealed that the lymphocytes constituted 48 per cent, monocytes 8 per cent, neutrophils 43 per cent and basophils 1 per cent.

Hikmet (164) reported 17,197 leucocytes per c.mm. of blood as the average for five animals. This investigator found the lymphocytes constituted 60.2 per cent of the total number of leucocytes, mononuclears 2.3 per cent, polymorphonuclears 31.1 per cent, eosinophils 5.5 per cent and basophils 0.9 per cent.

Gütig (163) studied the blood of 13 pigs and found marked variations in the total and differential counts. These findings showed for the total leucocyte count a mean of 21,500, with a maximum of 40,000 and a mini-

num of 10,000. The mean for the lymphocytes was 42.0 per cent, with a maximum of 65 per cent and a minimum of 21 per cent. The mononuclears averaged 3 per cent, with a maximum of 5 per cent and a minimum of 3 per cent. The polymorphonuclears averaged 52 per cent, with a maximum of 62.5 per cent and a minimum of 32 per cent. The eosinophils averaged 2 per cent and varied from 0.0 to 6.6 per cent. The basophils averaged 2 per cent and varied from 0.0 to 6.0 per cent.

The interesting feature of these results is that the polymorphonuclears form a greater proportion of the total number of leucocytes than do the lymphocytes. A wide range was found in the total number.

Lütje (170) reported 14,890 leucocytes per c.mm. of blood in pigs six weeks of age, compared with 18,600 for an older group. Differential counts were made on 22 pigs. His results were as follows: lymphocytes averaged 31 per cent and ranged between 37 per cent and 48 per cent, polymorphonuclears averaged 55.6 per cent and ranged between 48 and 60 per cent, eosinophils averaged 3.3 per cent and ranged between 0.0 and 10 per cent, basophils averaged 1 per cent and ranged between 0.0 and 4 per cent, transitionals averaged 4.7 per cent and ranged between 0.0 and 11 per cent.

It is interesting that this investigator found a larger number of polymorphonuclears than lymphocytes. In this respect Lütje agreed with Gütig.

King and Wilson (166) reported wide variations in cells of the leucocyte series, as observed in 43 normal pigs. A summary of their results is as follows: the total leucocyte count averaged 19,982, with a maximum of 29,000 and a minimum of 10,070; the lymphocytes averaged 54.2 per cent, with a maximum of 78 per cent and a minimum of 27.1 per cent; the large mononuclears averaged 3.7 per cent and varied between 0.0 and 22.1 per cent; the polymorphonuclears averaged 35.8 per cent, with a maximum of 72.2 per cent and a minimum of 16 per cent; the eosinophils averaged 4.5 per cent, with a maximum of 12.4 per cent and a minimum of 0.7 per cent; the transitionals averaged 0.4 per cent and varied from 0.0 to 4 per cent; the mast cells averaged 0.7 per cent and varied from 0.0 to 2.5 per cent.

It is seen that there is a wide variations in the total leucocyte counts. The lymphocytes here also form the greater part of the total number.

Dinwiddie (160) made observations on 16 pigs which he considered normal. The results of his work were as follows: the total leucocyte count averaged 11,840, with a maximum of 17,600 and a mean of 7,000. The mononuclears averaged 58 per cent, with a maximum of 77 per cent and a minimum of 38 per cent. The polymorphonuclears averaged 35 per cent, with a maximum of 75 per cent and a minimum of 24 per cent. The transitionals averaged 2.9 per cent and varied from 0.0 to 6 per cent. The eosinophils averaged 4 per cent and varied from 0.0 to 10 per cent. The mast cells averaged 0.0 per cent and varied from 0.0 to 2 per cent.

The total leucocyte count is lower than that given by most other investigators. Cells of the monocuclear type form the greater part of the total.

In a study of eight normal pigs Lewis and Shope (169) found the leucocyte count varied between 14,000 and 24,000 per c.mm. of blood. A later report by Lewis and Shope (168) gave 17,860 leucocytes as the average for six pigs. They also found 15 normal animals taken from the field, fluctuated between 14,000 and 26,500, with a mean of 21,800 leucocytes per



c.mm. Another group of 24 pigs from different herds showed counts between 19,000 and 35,000, with an average of 24,600 per c.mm.

### *Percentage of Corpuscles in Blood*

No reference was found in the literature concerning the percentage of corpuscles to plasma in swine blood. Howell (165, p. 420) stated that the proportion of corpuscles to plasma was 1 to 2. Smith (181, p. 4) stated that the plasma as a rule formed 66 per cent of the blood by volume. According to Starling (181, p. 685) plasma makes up 50 to 60 per cent of the total volume in normal human blood; about 55 per cent in horse blood; and 35 per cent in dog blood.

#### BLOOD OF CHOLERA-INFECTED SWINE

##### *Erythrocytes*

King and Wilson (166) reported the red corpuscles ranged from 2,800,000 to 9,056,000 per c.mm. of cholera-infected blood. The mean for 22 blood samples from cholera-infected pigs was 5,840,000 compared to 6,548,000 in the normal pigs they observed. Dinwiddie (160) examined 7 cholera-infected pigs and the erythrocytes ranged from 2,500,000 to 7,100,000 per c.mm. of blood. The average was 5,535,000 compared to 6,384,600 which he found in a normal group.

Lewis and co-workers (167) found 6,282,000 red blood corpuscles in a pig the day following inoculation with hog cholera virus and on the sixteenth day 3,480,000. Another pig that showed 5,740,000 the day before inoculation dropped to 2,000,000 on the fourteenth day following inoculation.

Naujek (174) stated that in light forms of the disease there was no change in the number of red corpuscles, but in acute or chronic forms of the disease the number dropped to as low as 3,000,000 per c.mm. Recent work of Lewis and Shope (169) showed that there was a slowly progressive anemia in hog cholera. One pig cited as typical of a group of eight was found to drop from 6,560,000 the day of inoculation to 5,875,000 on the eleventh day following and to 4,575,000 the twentieth day after inoculation. Later work reported by the same authors (168) confirmed the first report of a slight anemia. Five field cases of hog cholera averaged 4,913,000 erythrocytes per c.mm. of blood. Six experimental pigs averaging 6,825,000 erythrocytes per c.mm. before inoculation showed a decrease early in the disease.

Thorpe and Graham (183) found counts which ranged from 1,970,000 to 3,950,000, with an average of 2,886,000 in five cholera-infected pigs. Cases of hog cholera with complications likewise showed anemia.

##### *Hemoglobin*

King and Wilson (166) found the hemoglobin content in 22 cholera-infected pigs varied between 50 and 80 per cent, with a mean of 72.06 per cent. Lewis and co-workers (167) found the hemoglobin content was 80 and 90 per cent, respectively, in two cholera-infected pigs. Naujek (174) found that the hemoglobin dropped as low as 40 per cent in severe acute or



chronic cases of hog cholera. He also stated that the relationship between the hemoglobin content and erythrocyte count remained quite constant.

### *Leucocytes*

Regner (177) found that leucopenia occurs rapidly in hog cholera. Lütje (170) reported that a lymphocytosis occurred late in the disease. King and Wilson (166) noted a leucopenia in the 22 pigs studied. The results of their observations were as follows: the total leucocyte count averaged 15,515, with a maximum of 22,800 and a minimum of 7,200; the lymphocytes averaged 50.3 per cent, with a maximum of 74.2 per cent and a minimum of 16.3 per cent; the mononuclears averaged 10.5 per cent, with a maximum of 42 per cent and a minimum of 0.9 per cent; the polymorphonuclears averaged 31.8 per cent, with a maximum of 70 per cent and a minimum of 13.6 per cent; the eosinophils averaged 3.5 per cent and varied from 0.0 to 16 per cent; the basophils averaged 0.8 per cent and varied from 0.0 to 8.5 per cent; the transitionals averaged 3.5 per cent and varied from 0.0 to 18.1 per cent.

From these figures it can be seen that there are wide variations in the various counts, as is also true in the normal animals. The lymphocytes constitute the greater part of the total number of leucocytes. These authors state that a leucopenia was present in all cholera-infected pigs. The average decrease of leucocytes from the normal was 5,000 per c.mm. of blood. The average percentage of mononuclears was greater than in normal animals.

Dinwiddie (160) made observations on 13 cholera-infected pigs. His findings were as follows: the total leucocyte count averaged 6,509, with a maximum of 16,500 and a minimum of 1,800; the mononuclears averaged 56 per cent, with a maximum of 80 per cent and a minimum of 20 per cent; the polymorphonuclears averaged 35 per cent, with a maximum of 72 per cent and a minimum of 6 per cent; the transitionals averaged 7.3 per cent and varied from 0.0 to 16 per cent; the eosinophils averaged 6 per cent and varied from 0.0 to 10 per cent; the basophils averaged 1.1 per cent and varied from 0.0 to 2 per cent.

The total leucocyte count is much lower than for the normal. The mononuclear type cells make up the largest part of the total number. The transitional forms are increased markedly over the number in normal pigs.

Dinwiddie (160) described the transitional forms as those cells with irregular nuclei and with the cytoplasm showing a tendency to take eosin rather than basic stains. Many transitional or atypical forms were found very early in the course of the disease. He also stated that a leucopenia was present early in the disease.

Lewis and co-workers (167) inoculated two animals with hog cholera virus. One showed 20,000 leucocytes per c.mm. and the other 7,000, on the fourteenth day following inoculation. Naujek (174) stated that the leucocyte count decreased rapidly in cholera-infected pigs. He found that the lymphocytes increased and the polymorphonuclears decreased.

Lewis and Shope (169) found that leucocyte counts of 14,000 to 24,000 per c.mm. dropped to about 4,000 in twenty-four to forty-eight hours after the inoculation of pigs with hog cholera virus. If the disease developed a chronic course the count increased up to 8,000 or 10,000 by the seventeenth day. These authors stated that the field cases of hog cholera observed

ranged from 960 to 9,550 leucocytes per c.mm. of blood, with an average of 5,500. They concluded that a leucocyte count of 8,000 per c.mm. or less in three sick animals in a suspected herd indicated clearly that the condition was hog cholera. They further stated that hog cholera was the only acute infectious disease of swine characterized by a leucopenia.

Lewis and Shope made erythrocyte and leucocyte and differential counts on a pig before inoculation with hog cholera virus with the following results: erythrocytes 6,560,000; total leucocytes 23,700; polymorphonuclears 4,977; eosinophils 1,659; basophils none; lymphocytes 16,590; and mononuclears 474. On the seventh day after inoculation the following results were obtained: erythrocytes 5,830,000; total leucocytes 4,340; polymorphonuclears 1,606; eosinophils none; basophils none; lymphocytes 2,387; mononuclears 304; and questionable cells 43. On the thirteenth day after inoculation the erythrocytes numbered 5,000,000; the total leucocytes 2,900; the polymorphonuclears 145; the eosinophils 29; the basophils none; the lymphocytes 2,349; the mononuclears 377. On the twentieth day after inoculation the erythrocytes were 4,575,000; the total leucocytes 2,320; polymorphonuclears 70; eosinophils none; basophils 23; lymphocytes 2,065; mononuclears 162.

They state that these results, taken from one animal, are typical of the findings in eight pigs. A marked leucopenia and a moderate anemia may be noted. The percentage of polymorphonuclears is reduced more than that of the lymphocytes. The percentage of mononuclears increased during the course of the disease.

Lewis and Shope (168) later reported another study of cholera-infected blood. Six animals were inoculated with the virus of hog cholera. Five of these showed a leucopenia in 48 hours. By the time the temperature reaction appeared, leucocyte counts were as low as 2,000 to 4,000 per c.mm. of blood. The number of leucocytes began to increase slowly in 8 to 13 days if the animal did not die. Their observations on two animals before and after inoculation with the virus of hog cholera are as follows: in animal No. 432 before inoculation the total leucocyte count was 17,440; polymorphonuclears 30.5 per cent; eosinophils 8.5 per cent; basophils 1.5 per cent; lymphocytes 54.7 per cent; mononuclears 4 per cent. Nine days after inoculation the total leucocyte count was 2,900; polymorphonuclears 32 per cent; eosinophils and basophils none; lymphocytes 58 per cent; mononuclears 8 per cent; and endothelial cells 0.2 per cent.

In another animal, No. 434, before inoculation the total leucocyte count was 23,700; the polymorphonuclears 21 per cent; eosinophils 7 per cent; lymphocytes 70 per cent; and mononuclears 2 per cent. Nine days after inoculation the total leucocyte count was 3,440; the polymorphonuclears 20.9 per cent; lymphocytes 66.1 per cent; mononuclears 9 per cent; and endothelial cells 0.9 per cent.

The significant features of the above data are the marked leucopenia and the increase in mononuclear leucocytes.

Thorpe and Graham (183) found that 7,300 leucocytes per c.mm. of blood was the average in 25 pigs that showed lesions of hog cholera. Five uncomplicated cases of the disease averaged 8,700 per c.mm. One of these cases, however, showed a count of 22,500. They also stated that 17 cases of complicated hog cholera gave an average leucocyte count of 5,600 per c.mm.

## MATERIALS AND METHODS

## ANIMALS USED

The samples of normal blood were collected from various groups of pigs. Thirty samples were taken from pigs killed at the Iowa State College abattoir. Twenty-five of the samples were taken from animals that were the property of the Animal Husbandry Department, Iowa State College. Seven samples were procured from pigs belonging to the Department of Veterinary Research, Iowa State College. These pigs were normal so far as could be determined by a clinical examination. They ranged in weight from 50 to 320 pounds, about 50 per cent of the number weighing between 175 and 225 pounds.

The samples of cholera-infected blood were obtained from pigs that had been injected with hog cholera virus six to twelve days previously. These animals weighed between 40 and 90 pounds. All of the animals were autopsied to confirm the diagnosis of hog cholera. Twenty-eight blood samples were procured from pigs belonging to the Fort Dodge Serum Company. Seventeen of the blood samples were from animals that were the property of the United States Bureau of Animal Industry Experiment Station at Ames, Iowa. Ten of the blood samples were from animals that belonged to the Department of Veterinary Research, Iowa State College.

## BLOOD SAMPLES

Approximately 50 per cent of the blood samples were obtained by ear puncture, the counting and hemoglobin pipettes being filled immediately. Smears for differential counts were also secured at the same time. About 5 per cent of the samples were obtained by tail bleeding. The balance were obtained when the animals were killed by throat bleeding. In the latter two methods larger amounts of blood were taken and coagulation prevented by sodium citrate. The pipettes were filled and the smears made at the laboratory from the citrated blood.

The samples of cholera-infected blood were collected at the time that the pigs were killed in the procedure for the production of hog cholera virus. Eighty-five per cent of the cholera-infected blood samples were collected by throat bleeding, the remainder were taken direct from the ear vein before slaughter. Coagulation in the blood samples obtained by throat bleeding was prevented by sodium citrate.

## BLOOD COUNTING

Toisson's fluid was used as the diluent for counting erythrocytes. A few samples were counted in a 1-100 dilution, but the majority in a dilution of 1-200. The diluting fluid for the leucocytes consisted of a one per cent solution of acetic acid to which a small amount of gentian violet was added. In ten of the leucocyte counts dilutions of one to ten were used. In the remainder the blood was diluted one to twenty.

The American standard hemocytometer pipette was employed. The counting chambers were the improved Neubauer type.

The smears were air dried and stained with Wright's stain (Grübler's). The leucocytes were counted under the oil-immersion objective. Approximately 175 cells, on various parts of the field, were observed and recorded.



A microscope equipped with a mechanical stage was used so that the fields once counted would not be duplicated.

#### CLASSIFICATION OF LEUCOCYTES

Few investigators gave descriptions of cells of the leucocytic series in swine blood. The general descriptions given by Dinwiddie (160), Senftleben (179), Gütig (163), Giltner (162), and Palmer (176) are referred to.

Senftleben (179) stated that there were no essential differences between the cells of swine blood and those of the human blood. He stated that the polymorphonuclear leucocytes were cells, somewhat circular in shape, 10.2 to 13.3 $\mu$  in diameter. He further stated that the nucleus formed about one-third of the entire cell. The cytoplasm was described as similar to a pale pink veil and the nucleus appeared to lie on top of the cytoplasm.

Dinwiddie (160) stated that the polymorphonuclears did not show a distinct granulation. Gütig (163) noticed less deviation in the size of the polymorphonuclear cells of pig blood than in those of human blood. He also found that the cytoplasm stained red, in contrast to the pink color in the same type of cell of human origin. Palmer (176) stated that the several lobes of the nucleus nearly always assumed the shape of a spiral cord. Giltner (162) stated that if the time of staining was not carefully controlled the cell could not be often detected. Burnett (169, p. 36) stated that the nucleus was coarsely reticular, and that the small pin point like granules in the cytoplasm had a slight affinity for acid stains.

Gütig (163) and Senftleben (179) described the eosinophilic leucocytes as cells 10.5 to 13.75 $\mu$  in diameter. The cytoplasm was filled with large, shiny, red granules and the nucleus consisted of one or two lobes, more frequently two. Giltner (162) and Palmer (176) found in most cases a wide band connecting the two lobes of the nucleus. Giltner (162) stated that the granules of the cytoplasm were not as large, but were more numerous than those in the eosinophils of horse blood.

Senftleben (179) and Gütig (163) stated that the basophilic leucocytes were 10.8 and 15.3 $\mu$  in diameter. The nuclei were similar to those of the eosinophils in shape, but did not stain as deeply. The granules were large and distinctly basophilic. Gütig (163) found that these granules were soluble in aqueous or acid staining solutions. Dinwiddie (160) stated that these cells were often as numerous as the eosinophils.

According to Senftleben (179) there was no distinct difference between the monocytes and the lymphocytes. The mononuclear leucocytes had light staining nuclei, which were round, horseshoe or bean-shaped. They sometimes showed the degree of polymorphism seen in the eosinophils. The cytoplasm of the mononuclear leucocytes constituted a wider zone than in the large lymphocytes. The cytoplasm was a slate gray as contrasted with the dark blue or blue-green color of the lymphocytes. Giltner (162) also stated that the mononuclears were similar to the large lymphocytes. The nuclei were described as medium in size, bean-shaped and light blue in color.

Palmer (176) stated that the mononuclears were usually about the same size as the large lymphocytes, but occasionally they were five times the diameter of an erythrocyte. He stated that the differential features to be observed were: first, the great size of some of the cells; and second, the lighter staining nuclei and larger amount of visible cytoplasm as compared



to the lymphocytes. According to Burnett (159, p. 35) these cells were usually about twice the diameter of the erythrocytes. The nucleus, usually situated to one side of the cell, occupied about one-half of the entire cell. He also stated that both the cell body and nucleus were finely reticular and stained less deeply than the lymphocytes.

Dinwiddie (160) stated that the majority of the lymphocytes were about twice the diameter of erythrocytes. Giltner (162) divided the lymphocytes into two groups. The smaller cells were  $8.5\mu$  in diameter and the larger ones 11 to  $14\mu$  in diameter. The nucleus of the lymphocyte occupied relatively much less of the entire cell in the large type than in the small type. Burnett (159, p. 35) stated that the nucleus occupied nearly all of the cell mass. Both nucleus and cell body were coarsely reticular. He stated that with Wright's stain the nucleus was dark blue and the cytoplasm was a blue-green color.

The descriptions of the leucocytes in normal human blood as given by Downey (161, p. 570-573), Schilling (178, p. 125-133), Maximow (172, p. 61-66), and Todd and Sanford (184, p. 284-297) were studied. From these descriptions a classification was adopted and used throughout this investigation.

This classification is based upon the reaction of the leucocytes to Wright's stain. The classification is as follows: the polymorphonuclear leucocytes, which are also called heterophilic leucocytes, neutrophilic leucocytes or neutrophilic granulocytes, are cells with typical U, S or other odd shaped nuclei. The nuclei stain deeply with Wright's stain. The cytoplasm is faint pink in color, due to the granules having a weak affinity for acid. These cells are from one and one-half to two times the diameter of the red blood corpuscles.

The eosinophilic leucocytes or eosinophilic granular leucocytes are somewhat larger than the polymorphonuclear leucocytes. They have a polymorphic nucleus which is usually of two lobes. Large red granules are present in the cytoplasm, which make these cells easy to identify.

The cells commonly called basophils, basophilic leucocytes or mast cells are generally about the same size as the eosinophils, although they may be larger. The nuclei are polymorphic, but do not stain as deeply as those of the other granulocytes. The granules appear smaller than those of the eosinophils and take an intense basic stain. The nuclei are often obscured by the granules.

Lymphocytes are frequently divided into three groups according to size. Some authors classify the cells containing a single nucleus as large and small lymphocytes. Some classify these cells as lymphocytes and monocytes, whereas others class them into one group and designate them either as lymphocytes or monocytes. In this work the cells containing a single nucleus are classified as large or small lymphocytes, and mononuclear leucocytes.

Small lymphocytes are those mononuclear cells that range in size from one-half to the full diameter of an erythrocyte, and often to the size of a normal polymorphonuclear leucocyte. With Wright's stain the nuclei appear as solid dark masses in which the outline of the chromatin cannot be detected. Often no cytoplasm can be seen, but when observed it is a deep blue or blue-green color. No sharp line is drawn regarding the shape of the nucleus.

The large lymphocytes are larger than the polymorphonuclear leucocytes. Frequently the nuclei are about the size of those in the small lymphocytes, but a wide zone of cytoplasm is present. The cytoplasm assumes a deep blue or blue-green color with Wright's stain. Such a wide zone of cytoplasm is not observed in the smaller cells. These cells are differentiated from the mononuclear leucocytes by the darker staining qualities of the cells and by the clear cut nuclei, rather than by the size of the cells and shape of their nuclei. The chromatin material in the nuclei of the large lymphocytes often appears to resemble strings of beads. This arrangement of the chromatin is similar to that found in the nuclei of the polymorphonuclears.

Cells classified as mononuclear leucocytes, often called endothelial leucocytes, transitionals, monocytes or endotheliocytes, are generally the largest cells found in the blood. As a rule, the nuclei assume a light blue color with Wright's stain. In some instances, however, they stain deeply and are therefore difficult to differentiate from the large lymphocytes. The nuclei are most often bean-shaped, but they may be horseshoe-shaped or even approach the shape of the nuclei of the eosinophilic leucocytes. The chromatin appears as irregular thread-like masses. The fact that the nuclei are usually eccentrically located and occupy less space within the cells than they do in the large lymphocytes is an important factor in differentiation.

The cytoplasm of the mononuclear leucocytes has a rather characteristic slate gray color when stained with Wright's stain. Occasionally the cytoplasm assumes a pink tinge which makes it resemble that of the polymorphonuclear variety.

#### HEMOGLOBIN DETERMINATIONS

The hemoglobin content is stated in grams per hundred cubic centimeters of blood, as determined colorimetrically by the improved Newcomer method.

#### VOLUME PERCENTAGE OF CORPUSCLES

A volume of approximately 15 c.c. of blood was used in determining the volume percentage of corpuscles. The blood was mixed thoroughly in the collecting tube and then transferred to a 15 c.c. centrifuge tube. This blood was centrifuged for 20 minutes at 1,500 revolutions per minute, before readings were made.

#### RESULTS

##### BLOOD OF NORMAL SWINE

Observations were made and recorded on the morphology of the blood of 62 normal pigs that ranged in weight from 50 to 320 pounds. Fifty per cent of these pigs weighed between 175 and 225 pounds.

The erythrocyte counts in 51 normal pigs were found to range from 5,040,000 to 9,920,000 per c.mm. of blood, with a mean of  $7,907,156 \pm 106,560$ . The corpuscles in 24 blood samples ranged from 38 to 62 per cent of the total blood volume with a mean of  $47.8 \pm 0.96$ . The hemoglobin content as determined in 37 blood samples ranged from 5.9 to 17.4 grams per 100 c.c., the average being  $11.95 \pm 0.235$ .

The total leucocyte counts varied from 6,400 to 50,400 per c.m.. in 51 blood samples, the mean being  $21,523 \pm 661.9$ .

Fifty-seven smears made for differential counts showed wide variations in the different types of leucocytes. The polymorphonuclears ranged from 9.3 to 64.4 per cent, with a mean of  $36.16 \pm 1.12$ . The eosinophils ranged from 0 to 15.4 per cent, with a mean of  $4.09 \pm 0.303$ . The basophils varied between 0 and 2.4 per cent, the mean being  $0.42 \pm 0.06$ . The small lymphocytes ranged from 5.8 to 76.9 per cent of the total number of leucocytes and their mean was  $41.22 \pm 1.03$ . The large lymphocytes ranged from 0 to 58.3 per cent, with a mean of  $11.16 \pm 0.852$ . The mononuclear leucocytes ranged from 0 to 21.5 per cent, the mean being  $5.05 \pm 0.410$ .

#### BLOOD OF CHOLERA-INFECTED SWINE

Observations were made and recorded on the morphology of the blood of 57 cholera-infected pigs.

These pigs ranged in weight from 40 to 90 pounds.

The erythrocytes were found to range from 3,200,000 to 9,600,000 in 38 samples of cholera-infected blood, the mean being  $6,486,055 \pm 155,019$ . The volume of corpuscles in 54 samples varied from 25 to 62 per cent, with a mean of  $37.4 \pm 0.64$ . The hemoglobin in 56 samples of cholera-infected blood ranged from 6.22 to 14.03 grams per 100 c.c., with a mean of  $10.76 \pm 0.19$ .

The total leucocyte count in 56 samples of cholera-infected blood ranged from 2,600 to 86,400 per c.mm. of blood, the mean being  $10,422 \pm 1,359$ .

A differential leucocyte count on 54 samples of cholera-infected blood revealed wide variations in percentage counts of the different cells. The polymorphonuclears ranged from 1.1 to 76.3 per cent, with a mean of  $38.27 \pm 1.93$ . The eosinophils varied from 0 to 4.9 per cent, with a mean of  $0.31 \pm 0.045$ . The basophils ranged from 0 to 2.7 per cent, with a mean of  $0.064 \pm 0.02$ . The small lymphocytes ranged from 5.3 to 78.6 per cent, with a mean of  $33.02 \pm 1.97$ . The large lymphocytes ranged from 2.7 to 46.6 per cent of the total, with a mean of  $17.3 \pm 1.05$ . The mononuclear cells ranged from 0 to 64.2 per cent, with a mean of  $11.42 \pm 1.24$ .

#### DISCUSSION

##### NORMAL BLOOD

In a study of this nature there are always many factors that cannot be controlled by the investigator which may be responsible for the variations noted. The cellular elements of the blood may show variations with feeding, pregnancy, lactation and certain other normal processes. There are other variations associated with environment, such as temperature and altitude, which at times must be given consideration. It was thought that by making observations on the blood from a large number of animals, and subjecting the findings to statistical analysis that this would in a large measure compensate for the factors that could not be controlled.

The age of the animal is always a factor to be considered in observing the cellular elements of the blood of an individual. This is true of swine blood as shown by tSorch (182), Palmer (176), entleben (179), and Regner (177).

While reference is sometimes made to sex being a factor in blood counting, Storch (182), Welsch (185), and Palmer (176), yet it is relatively unimportant.



Some investigators question the collecting of samples of blood in sodium citrate or other anti-coagulants, believing that microscopic clots are always formed and that they may interfere with the obtaining of accurate results. A survey of the figures obtained in this work indicated no greater variations in counts from citrated blood samples than from those where the pipettes were filled direct.

The normal pigs were those that showed no apparent clinical evidence of disease. Such factors as parasitic infestations not severe enough to produce clinical symptoms, moderate metabolic derangements or other occult conditions may be responsible for some of the unusual variations encountered.

The erythrocytes are seen to vary greatly in number. The mean of 7,907,156 per c.mm. of blood for 51 pigs is much higher than the normal given for human blood. This count agrees with the results of Giltner (162), Lütje (170), Welsch (185), and Senftleben (179). It would seem reasonable to state that the number of erythrocytes in normal swine blood ranges between 7,500,000 and 8,500,000 per c.mm. of blood. The mean for 32 males was found to be 8,136,870 per c.mm. of blood, compared to 7,808,080 per c.mm. for the mean of 15 females. This difference is not significant.

The volumes of corpuscles and plasma are 48 and 52 per cent, respectively. There are variations that cannot be explained as the samples were all handled in the same manner. In all instances the blood was centrifuged for a sufficient time to completely separate the corpuscles from the plasma. In some instances the corpuscles settled out slowly and it was necessary to centrifuge a second time to insure complete separation.

The mean hemoglobin content of 11.95 grams per 100 c.c. of blood is lower than that for normal human blood. The hemoglobin determinations were made within an hour after the blood samples were collected. In the majority of cases the hemoglobin content ranged from 10.5 grams to 12.5 grams per 100 c.c. of blood.

The high count of leucocytes found in swine blood is an interesting feature. The mean of 21,523 leucocytes per c.mm. of blood for 51 animals is considerably higher than that given for other domestic animals or for man. Except for two counts of 40,000 in pig number 119, and 50,400 in pig number 120, which were considered high, the majority ranged from 17,000 to 25,000 per c.mm. These findings are in agreement with the reports of Giltner (162), Gütig (163), Palmer (176), Senftleben (179), Welsch (185), Hikmet (164), King and Wilson (166), and Lewis and Shope (168, 169) in that the majority of their counts were within this range. No specific explanation can be given for the two high counts. In both cases the percentage of polymorphonuclears is higher than the mean, which may indicate an occult infection, thus causing a leucocytosis.

A striking feature of the differential leucocyte count is the greater percentage of lymphocytes compared with the polymorphonuclears. This is in agreement with reports by Giltner (162), Palmer (176), Senftleben (179), Welsch (185), Hikmet (164), King and Wilson (166), and Dinwiddie (160). The small and large lymphocytes together make up 52.38 per cent of the total number of leucocytes compared to 36.6 per cent of polymorphonuclears. This relationship is readily noticed because in man and domestic mammals, with the exception of the ox and sheep, the polymorphonuclears are predominant.



The relationships of the mean number of eosinophils, basophils and mononuclear leucocytes to the total number of leucocytes are very similar to those found in other animals. Wide variations are found, however, in the eosinophils and mononuclears. The largest number of eosinophils, 15.4 per cent, was recorded in pig number 69. The largest number of mononuclears was 21.5 per cent in pig number 96.

The leucocytes in the various samples of normal blood were quite uniform in their characteristics. In pig number 129, however, all types of leucocytes were extremely large and in pig number 95 the eosinophils were about twice the ordinary size. In the blood of pig number 127 the mononuclears and polymorphonuclears were small but very distinct.

In many instances it was difficult to determine with certainty whether a cell should be classified as a mononuclear leucocyte or a large lymphocyte. The reason for this was the fact that the staining reactions failed to bring out the differential characteristics.

#### CHOLERA-INFECTED BLOOD

No individual weights were kept on pigs from which the cholera-infected blood samples were secured. They were, however, within the 40 to 90 pound limit, which is the United States Bureau of Animal Industry standard weight for virus pigs. About 75 per cent of the group were estimated to weigh between 65 and 80 pounds. Healthy pigs were inoculated with hog cholera virus, after which the temperature curves and clinical symptoms were observed daily to note the development and progress of the disease. The killing time varied somewhat, but was usually the sixth or seventh day following inoculation.

There is a wider range in the number of erythrocytes in cholera-infected blood than in normal blood, the mean of 6,486,055 per c.mm., however, is lower than the mean for normals. There are two possible reasons for the decrease. The corpuscles may have been injured by the virus and were degenerating more rapidly than normally, or the virus may have in some way interfered with the normal hematopoietic functions of the blood forming organs. A moderate anemia was reported by King and Wilson (166), Naujek (174), Lewis and Shope (168, 169), and Thorpe and Graham (183).

The mean volume of corpuscles was found to be 37 per cent in cholera-infected blood. This is a 10 per cent decrease from the mean volume of the normal blood. Again this decrease may be due to a rapid degeneration of the erythrocytes, or to injury to the hematopoietic organs. Also, there is the possibility of an increase of plasma which would reduce the volume of corpuscles in a given amount of serum. The decrease in volume percentage of corpuscles is not as marked as the decrease noted in the erythrocyte count.

The mean hemoglobin content as determined on 56 individual cholera-infected blood samples was 10.76 grams per 100 c.c. There was less variation in these samples, and the mean was lower than in the normal group. The decrease in hemoglobin is correlated with the decrease in erythrocyte count and the decrease in volume percentage of corpuscles in the cholera-infected blood. The hemoglobin content of the samples of cholera-infected blood obtained from the Fort Dodge Serum Company was generally lower than those from other sources. These samples were collected in sodium

citrate and determinations were made approximately six hours later. The hemoglobin content of cholera-infected blood samples from other sources was determined within an hour after having been procured. This time element may account for the lower hemoglobin content, which is quite uniform in the Fort Dodge samples.

The total leucocyte count showed rather wide variations, however, only eight of the 56 samples counted were higher than 12,000 per c.mm. of blood. Including these high counts, the mean of 56 cholera-infected pigs was found to be 10,422 per c.mm. No explanation is offered for the exceedingly high count of 86,400 in pig number 22. This count was checked by three individuals and it was thought advisable to report it even though it is entirely out of harmony with the other counts. Pig number 8c, which showed 68,000 leucocytes, also showed 66.1 per cent polymorphonuclears, which possibly indicated a secondary infection.

Only four of the 56 cholera-infected blood samples showed a leucocyte count higher than the mean of 21,524 established for the normal group. These high counts may have been due to a secondary infection. Frequently field cases of hog cholera are complicated with secondary infection.

The general leucopenia which was found in this study of hog cholera-infected blood agreed with the reports of Regner (177), King and Wilson (166), Dinwiddie (160), Lewis and Shope (168, 169), and Thorpe and Graham (183). Lewis and Shope (168) reported that this same type of leucopenia was observed in field cases of an acute nature. Thorpe and Graham (183) reported finding leucopenia occurring in hog cholera with complications as well as in cases diagnosed as pure hog cholera.

The result of these studies show that the numerical relationships of the various types of leucocytes are changed in cholera-infected blood. The mean for the polymorphonuclears shows an increase of 2.1 per cent over the mean of the normal; the small lymphocytes decrease 8.21 per cent and the large lymphocytes increase 6.09 per cent. The total number of lymphocytes is only 2 per cent lower than in the normal blood, but the proportion of the small lymphocytes and large lymphocytes has been changed.

The mononuclear leucocytes showed a wider range of variation than did the same type of cells in the normal blood. The mean of mononuclears increased 6.36 per cent over the mean number found in normal blood. King and Wilson (166) also found an increased number of mononuclear leucocytes in hog cholera-infected blood.

In some instances it was very difficult to determine whether a cell was a mononuclear leucocyte, a large lymphocyte or a polymorphonuclear leucocyte. It was decided to group these cells, according to their most apparent structural and staining characteristics, with the definite types rather than include a separate group for transitional or questionable forms.

Cells the size of mononuclear leucocytes often appeared, in which the nuclei were typically polymorphic and deeply stained. The cytoplasm on careful examination appeared to be faintly pink. These cells were classified as polymorphonuclears. If similar shaped cells were found having nuclei that stained a light blue and the cytoplasm gray, or faint pink, they were classified as mononuclears.

Large mononuclear type cells with dark staining nuclei and blue cytoplasm were designated as large lymphocytes. If the nuclei were light blue

and the cytoplasm gray or pink they were classified as mononuclear leucocytes.

In some instances it was evident that the leucocytes were degenerating. The nuclei were shattered in certain cells and in others the whole cell seemed to be involved. A few cells in such a condition were noted in smears wherein the majority of the cells were normal. This indicated that the cells were injured before the blood was drawn, and not in the process of preparation.

The numbers of eosinophils and basophils were reduced in the cholera-infected blood. This may have been due to direct injury or more likely to the fact that the total number of leucocytes were so markedly decreased.

The erythrocyte counts, the total leucocyte and differential leucocyte counts of the blood of cholera-infected pigs, obtained from various sources, were subjected to statistical analysis. The results are given in table 46.

It will be seen from table 46 that there are marked differences in the counts of some of the elements, while in others, there is very little. By this statistical analysis it is shown that only four of the variations cited are significant. In making these calculations the usual criterion is used, that is, if the mean difference is equal to or greater than three times the probable error of the mean difference the ratio is significant.

The outstanding features of this table are that the cholera-infected blood samples from the Fort Dodge Serum Company pigs are significantly lower in the mean number of total leucocytes than are those from the two other sources. Again the mean percentage of mononuclear leucocytes is significantly higher in the cholera-infected blood samples obtained from the pigs of the United States Bureau of Animal Industry Experiment Station than is that of the Fort Dodge group. Another significant feature is that the mean percentage of small lymphocytes is higher in the samples from the Fort Dodge pigs than in the samples obtained from the Veterinary Research Department.

There are two possible explanations for the erythrocyte and total leucocyte counts being lower in the blood samples from the Fort Dodge pigs. These pigs came from a region in North Dakota where hog cholera was not generally prevalent, hence the pigs likely had less resistance and were highly susceptible to the virus. The pigs in the other two groups were of Iowa origin and may have had some slight acquired immunity. Secondly, there was a possibility of a difference in the virulence of the strains of virus used.

#### NORMAL AND CHOLERA-INFECTED BLOOD COMPARED

The data collected on the normal blood and that on the cholera-infected blood were compared by statistical analysis. The differences between the means of normal pig blood and cholera-infected blood, the probable errors of these mean differences, and the ratio of each mean difference to its probable error are shown in table 47.

Again the usual criterion of a mean difference being equal to or greater than three times its probable error is used. The ratios which are significant are designated by *italics* in this table.

It is noted that in each case of the hemoglobin content, the erythrocyte count, and the total leucocyte count, that the ratios show the mean differences to be significantly higher, in favor of the normal pigs. An examination of the differential leucocyte count shows the mean differences of the

TABLE 46. *Mean erythrocyte and total and differential leucocyte counts of the blood of cholera-infected swine*

Source	Erythrocytes per c.mm. of blood	Leucocytes per c.mm. of blood	Differential count*					
			Polymorphonuclears	Eosinophils	Basophils	Small lymphocytes	Large lymphocytes	Mono-nuclears
Veterinary Research	6,776,000 ±317,158	17,178 ±3,999	47.09 ±5.06	0.65 ±0.29	0.37 ±0.197	18.34 ±3.14	16.69 ±4.45	16.76 ±4.61
U. S. B. A. I. Experiment Station	7,019,647 ±202,187	17,489 ±3,504	39.8 ±3.7	0.23 ±0.09	0.04 ±0.002	26.15 ±3.93	16.54 ±1.29	17.07 ±1.07
Fort Dodge Serum Company	5,598,177 ±271,940	4,609 ±165	37.01 ±2.2	0.26 ±0.069	0.3 ±0.002	38.36 ±2.13	18.84 ±1.39	5.87 ±0.67

\*Differential count in percentage.



TABLE 47. Comparison of hemoglobin and certain morphological elements in normal and cholera-infected blood of swine

Constituents	Normal Swine		Cholera Swine		Mean difference (—) signifies that the mean difference is in favor of cholera blood	Ratio
	No. of deter- mina- tions	Means grams per 100 c.c.	No. of deter- mina- tions	Means grams per 100 c.c.		
Hemoglobin	37	11.95 ± .24 grams per 100 c.c.	54	10.76 ± .190 grams per 100 c.c.	1.19 ± .302	3.9
Erythrocytes	51	7,907,157 ±106,560 per c.mm.	36	6,486,055 ±155,019 per c.mm.	1,421,101 ±188,112	7.5
White blood corpuscles	51	21,524 ±662 per c.mm.	53	10,422 ±1,359 per c.mm.	11,101 ±1,502	7.3
Polymorphonuclears	57	36.16% ± 1.2	48	38.26% ± 1.92	-2.1 ± 2.22	.9
Eosinophils	57	4.09% ± .30	48	.23% ± .045	3.859 ± .306	12.6
Basophils	57	.41% ± .06	48	.07% ± .02	.34 ± .06	5.1
Small lymphocytes	57	41.22% ± 1.03	48	33.01% ± 1.97	8.20 ± 2.22	3.6
Large lymphocytes	57	11.16% ± .85	48	17.25% ± 1.04	-6.09 ± 1.35	4.5
Mononuclears	57	5.05% ± .41	48	11.41% ± 1.24	-6.39 ± 1.30	4.3
Percentage of corpuscles	25	47.08% ± .96	52	37.38% ± .64	10.41 ± 1.15	8.9

eosinophils, basophils, and small lymphocytes to be significantly higher in the blood of normal pigs. The mean differences of the large lymphocytes and the mononuclear leucocytes are significantly higher in cholera-infected blood than in normal blood.

#### SUMMARY

A review of the literature is given on the morphology of the normal blood and of cholera-infected blood of swine. While it is noted there are a limited number of studies on the blood of normal swine, there are only a few references to the morphological changes in the blood of swine infected with hog cholera.

Studies were made on erythrocyte counts, volume percentage of corpuscles, hemoglobin content and total and differential leucocyte counts of the blood of normal and cholera-infected swine.

Mention is made of the sources of all blood samples. Sixty-two normal blood samples and 58 cholera-infected blood samples were used for this work.

The data accumulated from the observations on normal and cholera-infected blood are studied from the group standpoint. The individual findings are mentioned only when they are entirely out of harmony with the majority.

The data on the normal blood and the cholera-infected blood are compared to note the changes which occur as a result of hog cholera infection.

The hog cholera blood samples from various sources are compared.

All methods used are recognized standard laboratory procedures.

As an aid to the interpretation of the changes noted the figures were submitted to statistical analysis.

#### CONCLUSIONS

The mean erythrocyte count for 51 normal pigs was found to be 7,907,157 per c.mm. of blood, while that for 38 hog cholera-infected pigs was 6,486,055 per c.mm. The volume of corpuscles in the blood of 25 normal pigs was 47.08 per cent, whereas that for 54 cholera-infected pigs was 37.38 per cent. The mean hemoglobin content for 37 normal pigs was found to be 11.95 grams per 100 c.c. of blood, and that for 56 cholera-infected pigs 10.76 grams.

A moderate anemia was noted in the cholera-infected pigs. This was evidenced by a decrease in the erythrocyte count, volume percentage of corpuscles and hemoglobin content.

The mean total leucocyte count for 51 normal pigs was found to be 21,524 per c.mm. of blood, while that for 56 cholera-infected pigs was 10,422 per c.mm. The lymphocytes made up the greater part of the total number of leucocytes in both groups.

In a comparison of the differential counts of the normal and cholera-infected pigs, the most pronounced features were: the large lymphocytes were 6.09 per cent and the mononuclear leucocytes were 6.39 per cent higher in the blood of cholera-infected pigs than in that of normal pigs. The polymorphonuclear leucocytes were 2.1 per cent higher in the cholera-infected group. The small lymphocytes were 8.2 per cent, the eosinophils 3.85 per cent, and the basophils 0.34 per cent higher in the blood of normal

pigs than in that of cholera-infected pigs. The significance of these changes was not explained.

A leucocyte count considerably below the mean of 21,524 established for the normal pigs was found in all but 4 of the 56 cases examined, which were of acute, supposedly uncomplicated hog cholera. The degree of leucopenia in the cholera-infected pigs varied, but was most pronounced in the pigs originating from North Dakota.

In acute uncomplicated hog cholera, leucopenia is apparently sufficiently significant to warrant a leucocyte count as an aid to diagnosis. Field cases of hog cholera are commonly complicated by secondary infection, whereby the leucocytes may be variously influenced. Therefore the use of a leucocyte count as a routine measure, as an aid to diagnosis, necessitates extremely careful interpretation.

Further study, especially of field cases of hog cholera and possibly other acute diseases of swine, is necessary to determine positively the value of blood counts as an aid to the diagnosis of hog cholera.

#### LITERATURE CITED

157. ALLUMBAUGH, H. R.  
1929. A study of the blood of the mother and new born. *Proc. Soc. Exp. Biol. and Med.*, **26**:814-826.
158. BETHE, M.  
Beiträge zur Kenntnis der Zahl-und Massverhältnisse der roten Blutkörperchen. p. 6. *Med. Diss.*, Strassberg, 1891. Quoted by Welsch (185, pp. 38 and 40). Original not seen.
159. BURNETT, S. H.  
1908. The clinical pathology of the blood of domesticated animals. Taylor and Carpenter, Ithaca, New York. pp. 34-39.
160. DINWIDDIE, R. R.  
1914. Studies on the hematology of normal and cholera infected hogs. *Ark. Exp. Sta. Bul.* 120.
161. DOWNEY, HAL  
1930. Diseases of the blood. Textbook of pathology (by Bell, E. T.). Lea and Febiger, Philadelphia, pp. 570-573.
162. GILTNER, WARD  
1907. The histology and physiology of normal pig's blood. *Jour. Comp. Path. and Ther.*, **20**:18-23.
163. GÜTTIG, K.  
1907. Ein Beitrag zur Morphologie des Schweineblutes. *Archiv. f. mikrosk. Anat.*, **70**:629-693.
164. HIKMET, PERTEV  
1926. Die Blutplättchen beim gesunden und kranken Pferd, Hund and Schwein. *Archiv. f. wissenschaft. u. prakt. Tier.*, **55**:222-250.
165. HOWELL, W. H.  
1930. A text-book of physiology. W. B. Saunders Co., Philadelphia, p. 420.
166. KING, W. E., AND R. H. WILSON  
1910. Studies in hog cholera preventive treatment. Part II. Hematological studies. *Kan. Sta. Agr. Exp. Sta. Bul.* 171.
167. LEWIS, L. L., W. P. SCHULER, C. H. McELROY AND L. B. RITTER  
1914. Hog Cholera. *Okla. Agr. Exp. Sta. Bul.* 104.

168. ———, P. A., AND R. E. SHOPE  
1929. The blood in hog cholera. *Jour. Exp. Med.*, 50:719-737.
169. ——— AND ———  
1929. The study of the cells of the blood as an aid to the diagnosis of hog cholera. *Jour. Am. Vet. Med. Assoc.*, 74 (n.s. 27) :145-152.
170. LÜTJE, F.  
1911. Untersuchungen über die körperlichen Elemente des Blutes normaler und schweinepestkranker Schweine. *Inaug. Diss.*, Gießen.
171. MARLOFF, R.  
1919. Die früheren Zählungen der Erythrocyten im Blute verschiedener Tiere sind teilweise mit grossen Fehlern Behaftet. *Pflügers Arch. d. ges. Physiol.*, 175:355-370.
172. MAXIMOW, A. A.  
1930. A textbook of histology. W. B. Saunders Co., Philadelphia, pp. 61-66.
173. MÜLLER, G. A.  
1866. Beitrag zur Kenntnis des Oxyhämoglobins im Blute der Haussäugetiere und des Hausgeflügels. p. 36 *Philos. Diss.*, Erlangen. Quoted by Welsch (185, p. 39). Original not seen.
174. NAUJEK, W.  
1926. Das Blutbild und der Hämoglobinwert bei Virusschweinepest. *Deutsch. tierärztl. Wchnschr.*, 34:153-154.
175. PALMER, C. C.  
1917. Effects of muscular exercise and heat of the sun on the blood and body temperatures of normal pigs. *Jour. Agr. Res.*, 9:167-182.
176. ———  
1917. Morphology of normal pig's blood. *Jour. Agr. Res.*, 9:131-140.
177. REGNER, ALEXANDER  
1923. Ein Beitrag zum Blutbilde des gesunden und kranken Schweines und dessen Verwertung bei der Diagnose von Rotlauf, Schweinepest und Schweine-seuche. *Wien. tierärztl. Monatschr.*, 10:97-103.
178. SCHILLING, VIKTOR  
1929. The blood picture. C. V. Mosby Co., St. Louis, pp. 125-133.
179. SENTFLEBEN, OTTO  
1919. Das blutbild des gesunden Schweines. *Monaschr f. Prak. Tierheil.*, 30:289-313.
180. SMITH, F.  
1921. A manual of veterinary physiology. Alexander Eger, Chicago, p. 420.
181. STARLING, E. H.  
1930. Starling's principles of human physiology. Rev. and ed. by C. L. Evans. J. & A. Churchill, London, p. 688.
182. STORCH, A.  
Untersuchungen über den Blutkörperchen gehalt des Blutes des landwirtschaftlichen Haussäugetiere. *Inaug. Diss.*, Karlsruhe, 1904. Quoted by Gütig (163, p. 624). Original not seen.
183. THORPE, FRANK JR., AND R. GRAHAM  
1930. A study of the leucocyte changes in the blood of diseased swine. *Jour. Am. Vet. Med. Assoc.*, 78 (n.s. 30) :198-209.
184. TODD, J. S., AND A. H. SANFORD  
1929. Clinical diagnosis by laboratory methods. W. B. Saunders Co., Philadelphia, p. 284-297.



185. WELSCH, W.  
1923. Das Blut der Haustiere mit neuen Methoden untersucht. V. Untersuchung des Schweine-, Schaf-, und Ziegenblutes. Pflüger's arch. d. ges. Physiol., **198**:37-55.
186. WETZL, DR. J.  
1910. Klinische Blutuntersuchungen. Zeitsch. f. Tiermed., **14**:1-27.









# THE ACCURACY OF THE DILUTION METHOD OF ESTIMATING THE DENSITY OF A POPULATION OF MICRO-ORGANISMS

EDWARD S. ALLEN<sup>1</sup>

*From the Department of Mathematics, Iowa State College*

Accepted for publication December 1, 1931

A method widely used for estimating the number of bacteria, protozoa, etc., in a unit volume is the following. A set of  $n_1$  samples is taken, each containing  $a_1$  cc. of the original substance; then a set of  $n_2$  samples, each (because of an intervening dilution) containing the smaller amount  $a_2$  cc., and so on. It is assumed that even a single micro-organism would in a certain time make its presence known by reproduction, development of gas, or otherwise. The number of "fertile" tubes and the number of "sterile" ones in each set are observed, and from these observations the best estimate of the concentration of organisms in the original substance is made.

The theory of this method has been well developed by R. A. Fisher in his fundamental statistical memoir of 1922<sup>2</sup>. He treats not only of the best estimate of density, but also of the question of accuracy.

Tables for various choices of  $a_1$  and  $n_1$  have been computed, and are contained in works of Cutler, Crump and Sandon<sup>3</sup> and McCrady<sup>4</sup>. A recent unpublished thesis of N. R. Ziegler, written with the cooperation of H. O. Halvorson, gives yet other tables.

A number of authors draw attention to the rather large probable error involved in this method, and it seems worth while to study this question, chiefly by Fisher's method, in some more detail.

The questions which we should like to have answered are: "Given a certain result, in numbers of fertile tubes, what is the most probable number  $x$  of micro-organisms in a cubic centimeter of the original liquid? Between what figures may we assume, with a given degree of probability, that the true value lies?" Unfortunately, it is impossible to find answers of whose correctness we can be certain. The reason is that the calculation would require an assumption about a priori probabilities of all densities, and no such assumption is firmly founded, either on theory or on experiment. We could, most simply, assume equal ranges in the values of  $x$  to be equally probable. (This was done by Greenwood and Yule<sup>5</sup>, who obtained a formula substantially identical with formula (4) of this paper.)

---

<sup>1</sup>I wish to thank Professors R. E. Buchanan, R. A. Fisher and H. O. Halvorson for their encouragement and aid in this study. In particular, the tables compiled by Professor Halvorson and contained in the thesis, as yet unpublished, of Prof. Ziegler were of great service in the computations involved.

<sup>2</sup>R. A. Fisher. On the mathematical foundations of theoretical statistics, *Phil. Trans., Series A*, **222**, 1922. (Especially pp. 363-366.)

<sup>3</sup>D. W. Cutler, L. M. Crump and H. Sandon. A quantitative investigation of the bacterial and protozoan population of the soil, with an account of the protozoan fauna. *Phil. Trans., Series B*, **211**:317, 1922. The table was compiled by R. A. Fisher.

<sup>4</sup>M. H. McCrady. Tables for rapid interpretation of fermentation—tube results. *Pub. Health Journ.*, **9**:201, 1918.

<sup>5</sup>M. Greenwood and G. U. Yule. On the statistical interpretation of some bacteriological methods employed in water analysis. *Journ. of Hyg.*, **16**:36, 1917.

That is, it would be as reasonable to expect the number of bacteria in 1 cc. to be between 10 and 20 as between 1,000 and 1,010. One theoretical objection to this assumption is that it gives a finite probability for concentrations so high as to be physically impossible.

In view of the fact that micro-organisms often increase or decrease at a rate proportional to the number present, the logarithm of the concentration changing at a constant rate, we could assume equal ranges of  $\log x$  (to any base) equally probable. This would assume it equally probable that  $x$  lay between 10 and 20 and between 1,000 and 2,000. Perhaps this is reasonable enough in a certain range of values: it certainly leads to absurd conclusions for both high and low concentrations.

The inability to define a priori probability reliably, as well as the tedious nature of the computation which would lead from any such definition to answers to our questions, has led Fisher and others to ask, not for the  $x$  of maximum probability, but for that of maximum "likelihood."

Corresponding to every true concentration  $x$ , each possible experimental result—i. e., each number of fertile tubes in a given set—has a perfectly definite probability; this is proportional to what is called the likelihood of  $x$  for the experimental result. All tables used show that value for  $x$  which has maximum likelihood. Let us illustrate with an example. McCrady, taking 5 tubes with 10 cc. each of the original substance, 5 with 1 cc. each, 5 with .1 cc. each, and assuming that 3, 1 and 2, respectively, have micro-organisms, assigns to  $x$  the value .17. We should like to be able to infer that it is more probable that the true  $x$  lies between .165 and .175 than that it lies, let us say, between .145 and .155. This the method of computation does not permit us to say. The fact is this—if  $x$  is .17, then the probability of obtaining 3, 1, 2 is a certain number; if  $x$  is any other number of hundredths, the probability of obtaining 3, 1, 2 is smaller. Clearly this is not quite the information desired; but the error introduced by the substitution is inconsiderable, compared with the uncertainty inherent in the method under discussion.

Before proceeding to the mathematics used for the construction of tables and for the estimation of probable error, a very simple consideration will give an upper bound for the precision to be expected. As a particular case, suppose there are three sets of tubes, containing respectively 8, 1,  $\frac{1}{8}$  cc. of the original substance. A typical situation is that in which each of the first set is fertile and each of the last sterile. In such a case, we shall usually find every tube more concentrated than one in the first set to be fertile, and every tube more diluted than one in the last set sterile.

We will particularize further, supposing 16 tubes in each set. The possible records of experiment are 16, 16, 0; 16, 15, 0; 16, 14, 0; . . . ; 16, 1, 0; 16, 0, 0. It is clear that  $x$  indicated by the first result will be 8 times that indicated by the last if the number of dilutions is infinite. Roughly, we may expect the intermediate values of  $x$  to be in geometric progression, the common ratio being  $^{16}\sqrt{8} = 1.14$ . At the very best—if each value of  $x$  could yield but one of these experimental results—we should have to attach to any estimate of  $x$  an uncertain multiplier somewhere between  $(1.14)^{\frac{1}{2}}$  and  $(1.14)^{-\frac{1}{2}}$ . This multiplier can be forced closer to 1 in two ways—by a smaller ratio of dilution (it was 8 here) and by a greater number of tubes in each set. The ratio (1.14) used here would be replaced by  $^5\sqrt{10} = 1.58$  in McCrady's best tables, and  $\sqrt{2} = 1.41$  in those of Cutler, Crump and Sandon.

Can this point of view help us to decide on the best ratio of dilution and number of tubes per set, if the range of concentrations to be explored and the total number of tubes in the experiment are both given? It turns out that it gives no such information. If the uncertainty factor (corresponding to 1.14 in the preceding example) is  $f$ , and the total number of tubes available is  $n$ , the range of concentrations which they will explore is  $f^n$ , regardless of the manner in which  $n$  is factored to give the number of sets and the number of tubes in a set. Other considerations will be needed to decide the best choice of these factors.

Let us now proceed to the question of maximum likelihood. We assume several sets of tubes, the number in the first set being  $n_1$ , that in the second  $n_2$ , and so on. Each of the first set shall have  $a_1$  cc. of the original fluid, each of the second  $a_2$  cc., etc. If the number of organisms per cc. in the original fluid is  $x$ , we may expect  $a_1x$  of them in each tube of the first set,  $a_2x$  in each of the second . . . . In order to find the probability that a particular tube of the  $i$ th set is sterile, we suppose that each micro-organism occupies  $1/m$  of its volume—in other words, that there are  $m$  such units of volume in the tube. The probability that a particular unit should be occupied by an organism is, then,  $a_1x/m$ , the probability of its having sterile fluid  $1 - a_1x/m$ . The probability that all  $m$  units are sterile is then the product of the probabilities for the separate ones—that is,

$$\left(1 - \frac{a_1x}{m}\right)^m = \left[\left(1 - \frac{a_1x}{m}\right)^{-\frac{m}{a_1x}}\right]^{-a_1x} \quad (1)$$

Now, by the definition of  $e$ ,  $\lim_{z \rightarrow \infty} (1 + 1/z)^z$ —this constant is the approximate value of the bracket. The probability then that a particular tube is sterile<sup>6</sup> is  $e^{-a_1x}$ , and the probability of fertility  $1 - e^{-a_1x}$ . If, of the  $n_1$  tubes of the  $i$ th set,  $p_i$  particular ones are to be fertile and the rest sterile, the probability of this result is  $(e^{-a_1x})^{n_1-p_i} (1 - e^{-a_1x})^{p_i}$ . The number of ways of selecting the  $p_i$  fertile tubes is

$$\binom{n_1}{p_i} = \frac{n_1!}{p_i!(n_1 - p_i)!}$$

and so the probability that, for a given  $x$ , the experiment should result as it does—with  $p_1$  fertile tubes in the first set,  $p_2$  in the second, and so on—is the product of such factors;

$$P = \Pi \binom{n_i}{p_i} (e^{-a_i x})^{n_i - p_i} (1 - e^{-a_i x})^{p_i} \quad (2)$$

Here  $\Pi$  indicates "the product of factors of type." It is this product which is to be made as large as possible by an appropriate choice of  $x$ . If it is maximum, its logarithm is also maximum, and the derivative of the latter is zero. The resulting equation (where  $\Sigma$  means "the sum of of terms of type") is

<sup>6</sup>Fisher. loc. cit., p. 363.

$$\sum \frac{a_i p_i}{1 - e^{-a_i x}} = \sum a_i n_i \quad (3)$$

This will yield those tables which have been computed, except McCrady's—and even there, where another formula is used, the difference in results is insignificant.

If we knew equal ranges of value of  $x$  to be equally probable *a priori*, or for that matter, if any other distribution of *a priori* probability were known to be correct, formula (2) would yield full information on the inferences to be drawn from any experimental result. If, for example, we do assume equal probability (before the experiment) for equal ranges of  $x$ , then the probability, after the experiment, that  $x$  lie between any two non-negative numbers,  $x_1$  and  $x_2$ , is

$$\frac{\int_{x_1}^{x_2} P dx}{\int_0^{\infty} P dx} \quad (4)$$

In justification of this statement, let us recall that  $P$  depends both on the true concentration  $x$  and on the experimental result  $p_1, p_2 \dots$ . The assumption of equal *a priori* probability means that, of a large number of experiments of the type considered, as many will be performed on concentrations between  $a$  and  $a + \Delta x$  as on those between  $b$  and  $b + \Delta x$  ( $a$  and  $b$  any two positive numbers), and that the number in each case is proportional to  $\Delta x$ . The number of concentrations between  $x$  and  $x + \Delta x$  which result in  $p_1, p_2, \dots$  fertile tubes is approximately proportional to  $P(x; p_1, p_2, \dots) \Delta x$ , and the approximation approaches exactness as the number of experiments increases and  $\Delta x$  approaches 0. The number of all cases—for all  $x$ 's—resulting in  $p_1, p_2 \dots$  is proportional to

$$\lim_{\Delta x \rightarrow 0} \sum_{x=0}^{\infty} P \Delta x = \int_0^{\infty} P dx,$$

and the probability that the true  $x$  lies between  $x_1$  and  $x_2$  is the fraction (4). If the number of fertile tubes is not very small, there are many terms to be integrated, and the labor could only be justified if the underlying assumption were known to be correct. We will, then, give but two examples of its application—not so much for the information they yield as for the sake of comparison with results obtained by the method of maximum likelihood.

The first example corresponds to the maximum contamination permitted by the U. S. Treasury Standard in waters supplied by common carriers<sup>7</sup>.

<sup>7</sup>U. S. Pub. Health Rept. 1914, p. 2959.

See, also, discussion by McCrady, Journ. Inf. Dis., 17:203, 1915.



TABLE 1a

$$\begin{aligned} a_1 &= 10, & a_2 &= 1, & a_3 &= .1 \\ n_1 &= n_2 = n_3 &= 5 \\ p_1 &= 1, & p_2 &= 0, & p_3 &= 0 \end{aligned}$$

$x$	Probability that no. bacteria per cc. exceeds $x$
.005	.970
.01	.908
.02	.735
.03	.561
.04	.403
.05	.287
.06	.199
.07	.136

.02 is, approximately, the number which satisfied (3); yet it seems almost three times as probable that  $x$  lies above that number as that it lies below it.

If  $p_1 = 5$ ,  $p_2 = 2$ ,  $p_3 = 2$ , the other data being the same as before, we have the following information.

TABLE 1b

$x$	Probability that no. bacteria exceeds $x$
.6	.894
1.0	.485
2.0	.087

In this case the solution of (3) is .95, and the probability that this number is exceeded turns out to be very close to 50 per cent.

If, now, we leave the attempt to estimate the precision of the dilution method by integration, we will take the  $x$  of maximum likelihood and then, using Fisher's method<sup>s</sup>, determine the standard deviation of the natural logarithm of  $x$  on the assumption that the values of this logarithm have normal distribution. For two reasons this is likely to be nearer the truth than the assumption of normal distribution of  $x$  itself. One is the fact that  $\log x$  extends from  $-\infty$  to  $\infty$ , so that symmetric distribution is possible; the other, that growth or destruction of bacteria generally proceeds according to exponential laws. It is, in addition, more convenient to have the relative deviation from the most likely value of  $x$  than the absolute deviation to be expected.

If the chance that a variable lie between  $\theta_1$  and  $\theta_2 + d\theta$  is

$$\Phi = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(\theta_2 - \theta_1)^2}{2\sigma^2}} d\theta, \quad (5)$$

--the expression for normal probability distribution with  $\theta_1$  as mode and  $\sigma$  as standard deviation--then

<sup>s</sup>Fisher. loc. cit., pp 328, 364.

$$\ln \Phi = \ln \left( \frac{1}{\sigma \sqrt{2\pi}} d\theta \right) - \frac{(\theta_1 - \theta)^2}{2\sigma^2}$$

$$\frac{\delta \ln \Phi}{\delta \theta} = \frac{\theta_1 - \theta}{\sigma^2}$$

$$\frac{\delta^2 \ln \Phi}{\delta \theta^2} = -\frac{1}{\sigma^2} \quad (6)$$

a formula permitting easy computation of  $\sigma$ .  
For us,  $\theta = \ln x$ .

We have already obtained  $\frac{\delta \ln \Phi}{\delta \ln x} = 0$  in the form (3), and a second differentiation, if we make use of that equation, yields

$$\frac{1}{\sigma^2} = x^2 \sum \frac{a_i^2 p_i e^{-a_i x}}{(1 - e^{-a_i x})^2} \quad (7)$$

This gives a value of  $\sigma$  such that, in 68.27 per cent of the cases where the experimental results are as found, the true value of  $\ln x$  will differ from the most likely one by no more than  $\sigma$ . The ratio between the true value of  $x$  and the most likely one is then between  $e^{-\sigma}$  and  $e^{\sigma}$ .

Before tabulating the results of this formula for various experimental arrangements, let us note, with Fisher, that if, in this formula, we replace the actual values of  $p_i$  by the average values to be expected for the given  $x$ , there results the simpler formula

$$\left( \frac{1}{\sigma^2} \right)_{av.} = x^2 \sum \frac{n_i a_i^2 e^{-a_i x}}{1 - e^{-a_i x}} \quad (8)$$

One result to be read off is that the multiplication of the numbers in all sets by any positive constant ( $k$ ) results in the division of  $\sigma$  by  $\sqrt{k}$ .

The first application of (7) will be to McCrady's most complete table—that for five tubes in each of three sets.

TABLE 2a

$$a_1 = 10, \quad a_2 = 1, \quad a_3 = .1$$

$$n_1 = n_2 = n_3 = 5$$

$p_1$	$p_2$	$p_3$	$\sum p_i$	$x$	$\sigma$	$e^{\sigma}$
0	0	1	1	.02	1.000	2.72
0	1	0	1	.02	.99	2.69
0	0	0	1	.02	1.005	2.74
0	0	2	2	.04	.708	2.03
0	1	1	2	.04	.708	2.03

$P_1$	$P_2$	$P_3$	$\Sigma p_i$	$x$	$\sigma$	$e\sigma$
0	2	0	2	.04	.709	2.03
1	0	1	2	.04	.707	2.03
1	1	0	2	.04	.710	2.04
2	0	0	2	.05	.682	1.98
0	1	2	3	.06	.574	1.78
0	2	1	3	.06	.576	1.78
0	3	0	3	.06	.579	1.79
1	0	2	3	.06	.576	1.78
1	1	1	3	.06	.579	1.79
1	2	0	3	.06	.581	1.79
2	0	1	3	.07	.586	1.80
2	1	0	3	.07	.587	1.80
3	0	0	3	.08	.611	1.84
1	0	3	4	.08	.504	1.66
1	1	2	4	.08	.499	1.65
1	2	1	4	.08	.494	1.64
1	3	0	4	.08	.493	1.64
2	0	2	4	.09	.509	1.67
2	1	1	4	.09	.509	1.67
2	2	0	4	.09	.509	1.67
3	0	1	4	.11	.514	1.67
3	1	0	4	.11	.520	1.69
4	0	0	4	.13	.537	1.71
1	2	2	5	.10	.451	1.57
1	3	1	5	.10	.451	1.57
1	4	0	5	.11	.452	1.57
2	0	3	5	.12	.458	1.58
2	1	2	5	.12	.458	1.58
2	2	1	5	.12	.458	1.58
2	3	0	5	.12	.458	1.58
3	0	2	5	.14	.459	1.58
3	1	1	5	.14	.470	1.60
3	2	0	5	.14	.470	1.60
4	0	1	5	.17	.490	1.64
4	1	0	5	.17	.492	1.64
5	0	0	5	.25	.580	1.79
2	2	2	6	.14	.419	1.52
2	3	1	6	.14	.419	1.52
2	4	0	6	.14	.419	1.52
3	1	2	6	.17	.432	1.54
3	2	1	6	.17	.432	1.54
3	3	0	6	.17	.432	1.54
4	0	2	6	.20	.452	1.57
4	1	1	6	.20	.454	1.57
4	2	0	6	.20	.454	1.57
5	0	1	6	.30	.535	1.71
5	1	0	6	.35	.581	1.79
3	1	3	7	.20	.404	1.50
3	2	2	7	.20	.404	1.50
3	3	1	7	.20	.404	1.50
3	4	0	7	.20	.404	1.50
4	0	3	7	.25	.430	1.54
4	1	2	7	.25	.430	1.54
4	2	1	7	.25	.431	1.54
4	3	0	7	.25	.431	1.54
5	0	2	7	.40	.535	1.71
5	1	1	7	.45	.565	1.76
5	2	0	7	.50	.598	1.82

$P_1$	$P_2$	$P_3$	$\Sigma p_i$	$x$	$\sigma$	$e\sigma$
3	4	1	8	.25	.369	1.45
3	5	0	8	.25	.369	1.45
4	2	2	8	.30	.410	1.51
4	3	1	8	.30	.409	1.51
4	4	0	8	.35	.425	1.56
5	0	3	8	.60	.543	1.72
5	1	2	8	.60	.545	1.73
5	2	1	8	.70	.565	1.76
5	3	0	8	.80	.592	1.81
4	3	2	9	.40	.402	1.50
4	4	1	9	.40	.402	1.50
4	5	0	9	.40	.402	1.50
5	0	4	9	.75	.491	1.63
5	1	3	9	.85	.500	1.65
5	2	2	9	.95	.511	1.67
5	3	1	9	1.10	.519	1.68
5	4	0	9	1.30	.537	1.72
4	5	1	10	.50	.390	1.48
5	2	3	10	1.20	.460	1.59
5	3	2	10	1.40	.470	1.60
5	4	1	10	1.70	.490	1.63
5	5	0	10	2.50	.578	1.78
5	2	4	11	1.50	.420	1.52
5	3	3	11	1.75	.434	1.55
5	4	2	11	2.50	.500	1.65
5	5	1	11	3.50	.581	1.79
5	2	5	12	1.75	.390	1.48
5	3	4	12	2.0	.382	1.53
5	4	3	12	3.0	.454	1.58
5	5	2	12	6.0	.648	1.92
5	3	5	13	2.5	.368	1.45
5	4	4	13	3.5	.425	1.53
5	5	3	13	9.0	.586	1.80
5	4	5	14	4.5	.415	1.51
5	5	4	14	16.0	.555	1.74

McCrary's results have been used in the first five columns, the various cases, however, being rearranged in the order of the total number of fertile tubes. The values of  $x$  below .20 are correct to the nearest hundredth, those between .20 and 2.0 to the nearest .05, those above 2 to the nearest .5. The last figure of  $\sigma$  given is, then, scarcely reliable.

Since the error may be expected to be below the standard deviation in 68.27 per cent of the cases (a well known property of the standard deviation), the table tells us that, in this fraction of the cases in which the numbers of fertile tubes are 3, 2, 1, respectively, the number of bacteria per cc. is between (.17) (1.54) and (.17) (1.54)<sup>-1</sup>, i. e., between .26 and .11. Should we wish to know the bounds which are not exceeded in 90 per cent of the cases we may use a table of values of the definite probability integral<sup>9</sup>. There we find that a change from 68.27 per cent to 90 per cent means a multiplication of  $\sigma$  by 1.645.  $\sigma$  is replaced by .710,  $e\sigma$  by 2.04, and

<sup>9</sup>e. g., that given in Rugg: Statistical methods applied to education, p. 391, and reproduced in Chaddock: Principles and methods of statistics, Appendix D.



the bounds within which  $x$  remains in 90 per cent of the cases are .35 and .08.

We may note in table 2a:

- (1) a wide variation in the most likely  $x$  among the cases with the same total number of fertile tubes;
- (2) an increase in this  $x$  as the tubes found fertile pass to the sets with higher concentration;
- (3) a corresponding decrease in precision (increase in  $\sigma$ );
- (4) except for cases with very few fertile tubes, lack of dependence between the total number of fertile tubes and the standard deviation.

$\sigma$  could of course be decreased by an increase of the number of tubes per set, the ratio of decrease of  $\sigma$  being the square root of the ratio of increase of  $n_1$  ( $p_1/n_1$  remaining constant). For instance, if there were 20 tubes in each set, and 12, 8, 4, respectively, were fertile, the most likely  $x$  would still be .17.  $\sigma$  would shrink from .432 to half that number—.216,  $e^\sigma$  from 1.54 to its square root, 1.24. The range of 68.27 per cent probability would extend from .21 to .14, that of 90 per cent probability from .24 to .12.

In order to see what decrease in deviation could be obtained by the use of smaller dilution intervals, I have computed a similar table, assuming seven sets of three tubes each, the dilution ratio being 2. The table is fragmentary, containing only data for which the highest concentration (and presumably all higher) shows all tubes fertile, while the lowest (and presumably all lower ones) shows all sterile; furthermore, most of the space is given to cases in which the number of fertile tubes is 10, 11 or 12. If the table were to be used in practice, this would be no great handicap; for if there were more than seven dilutions decreasing in concentration by the factor  $\frac{1}{2}$ , we should usually have at most five consecutive sets neither all fertile nor all sterile. The number of cc. of the original solution in each tube at the middle of this series can be taken as the unit, and the table used accordingly. Thus, if we have

Concentrations .....	32	16	8	4	2	1	1/2	1/4	1/8	1/16
No. fertile tubes .....	3	3	2	0	1	1	0	0	0	0

We could use the line of the table reading

3 3 2 0 1 1 0.

The middle 0 being now the number of 4 cc. tubes, the most likely number of bacteria per cc. is not .56, but one-fourth of that number, namely .14.

The table covers only the most probable cases for which the number of fertile tubes is 10, 11, 12. However, other cases in this range can be solved approximately by the empirical formula

$$\log_{10} x = .180 p_2 + .134 p_3 + .034 p_4 + .081 p_5 + .075 p_6 + 8.770 - 10 \quad (9)$$

TABLE 2b

$$a_1 = 8, a_2 = 4, a_3 = 2, a_4 = 1, a_5 = \frac{1}{2}, a_6 = \frac{1}{4}, a_7 = \frac{1}{8}$$

$$n_1 = n_2 = \dots = 3$$

$$p_1 = 3 \quad p_7 = 0$$

$p_2$	$p_3$	$p_4$	$p_5$	$p_6$	$\Sigma p_i$	$x$	$\sigma$	$e\sigma$
0	0	0	0	0	3	.088	.591	1.81
1	0	0	0	0	4	.127	.516	1.68
1	1	0	0	0	5	.168	.470	1.60
2	1	0	0	0	6	.236	.444	1.56
2	2	0	0	0	7	.286	.422	1.53
2	2	0	1	0	8	.342	.404	1.50
2	3	0	1	0	9	.42	.388	1.47
2	2	1	2	0	10	.48	.370	1.45
2	2	2	1	0	10	.49	.373	1.45
2	2	3	0	0	10	.50	.374	1.45
2	3	1	1	0	10	.51	.379	1.46
2	3	2	0	0	10	.53	.383	1.47
3	1	2	1	0	10	.55	.385	1.47
3	2	0	1	1	10	.56	.406	1.50
3	2	1	0	1	10	.57	.398	1.49
3	2	1	1	0	10	.58	.397	1.49
3	2	2	0	0	10	.60	.404	1.50
3	3	0	1	0	10	.62	.410	1.51
3	3	1	0	0	10	.64	.417	1.52
2	3	2	0	1	11	.61	.370	1.45
2	3	2	1	0	11	.62	.371	1.45
3	2	1	1	1	11	.69	.387	1.47
3	2	1	2	0	11	.69	.387	1.47
3	2	2	0	1	11	.71	.390	1.48
3	2	2	1	0	11	.72	.394	1.48
3	2	3	0	0	11	.74	.398	1.49
3	3	1	0	1	11	.76	.415	1.51
3	3	1	1	0	11	.78	.407	1.50
3	3	2	0	0	11	.81	.415	1.51
2	3	2	2	0	12	.72	.348	1.42
2	3	3	1	0	12	.74	.361	1.43
3	2	2	2	0	12	.86	.383	1.47
3	2	3	0	1	12	.88	.378	1.45
3	2	3	1	0	12	.90	.392	1.48
3	3	1	1	1	12	.93	.416	1.51
3	3	1	2	0	12	.95	.406	1.50
3	3	2	0	1	12	.97	.415	1.51
3	3	2	1	0	12	1.00	.410	1.51
3	3	3	0	0	12	1.06	.420	1.52
3	3	2	1	1	13	1.21	.403	1.50
3	3	2	2	1	14	1.50	.398	1.49
3	3	3	2	1	15	2.14	.429	1.54
3	3	3	2	2	16	2.71	.414	1.52
3	3	3	3	2	17	4.37	.476	1.61
3	3	3	3	3	18	5.46	.465	1.60

It will be seen that the standard deviations are systematically lower than those in table 2a. It might, however, be questioned whether this is due to the decrease in dilution ratio, to the increased number of tubes, or to the decreased range. To answer this question, I have compiled the following brief table, in which the same number of tubes and the same range are postulated as in table 2b, but the dilution range is increased to 8.

TABLE 2c

$$\begin{array}{l} a_1=8 \quad a_2=1 \quad a_3=\frac{1}{8} \\ n_1=n_2=n_3=7 \\ p_1=7 \quad p_3=0 \end{array}$$

$p_2$	$\Sigma p_i$	$x$	$\sigma$	$e\sigma$
0	7	.26	.459	1.53
1	8	.33	.446	1.56
3	9	.42	.421	1.52
3	10	.55	.464	1.59
4	11	.73	.470	1.60
5	12	1.01	.459	1.58
6	13	1.44	.505	1.66
7	14	2.20	.516	1.68

The standard deviations in table 2b are, in the main, smaller. There is a distinct gain in precision through the use of more frequent dilutions. If the experimenter, in the last case, wishes to attain the precision of the preceding one, he should increase the number of tubes per set by approximately 50 per cent.

A brief examination of the cases in which the method of integration was used shows somewhat lower estimates of the standard deviation than the corresponding ones in table 2a, but of the same order of magnitude. Or we may compare percentage probabilities. In tables 1a and 1b we found that, for 1, 0, 0 fertile tubes there was 83.4 per cent probability of an  $x$  between .005 and .07; and, for 5, 2, 2, 80.7 per cent of an  $x$  between .6 and 2.0. If, now, we use the  $\sigma$  of table 2a, and avail ourselves of published values of the probability integral, we find these percentages replaced by 80.0 per cent and 71.2 per cent, respectively. In these cases, at least, the assumption of equal distribution of probability of  $x$  leads to a slightly increased chance that the true concentration lies within a limited range.

It is interesting to see how the standard deviation can be reduced to the neighborhood of 5 per cent. In table 2b the values of  $\sigma_{1nX}$  are about .40. A reduction to .05 would call for an increase in number of tubes by  $8^2$ —so that the total number would no longer be 21, but over 1,000. It may be recalled that Wilson<sup>10</sup> claims a standard deviation as low as 5 per cent for an average of three tubes, while Stein<sup>11</sup> does not claim any value below 12 per cent, even when greatest care is used.

All the discussion thus far has assumed the exploration of a range of concentrations, the ratio of whose extreme values is not above 100. In

<sup>10</sup>G. S. Wilson. The proportion of viable bacteria in young cultures with especial reference to the technique employed in counting. *Journ. of Bact.*, 7:405, 1922.

<sup>11</sup>M. F. Stein. A critical study of the bacterial count in water and sewage. *Am. Journ. Pub. Health*, 8:820, 1918.

cases of total ignorance as to the number of organisms, a much larger range must accordingly be employed. If it is possible to repeat the experiment, or if the condition of the culture will remain fairly constant until a first sample has been tested, it would be well to discover the general neighborhood of  $x$  by the use of a few tests at dilutions wide apart, and to follow with a more intensive exploration the neighborhood where sterile tubes begin to appear.

As Fisher has pointed out<sup>12</sup>, the use of elaborate tables may be replaced by the count of the total number of fertile tubes. For equal efficiency, the number of tubes used must be increased by one-seventh if the dilution ratio is 2, the efficiency of this method being 87.71 per cent.

#### CONCLUSIONS

The dilution method of estimating the number of micro-organisms in unit volume, while reliable, involves a high probable error, if a moderate number of tubes are used.

For a given total number of tubes and a given range of concentrations, a low dilution ratio gives somewhat smaller errors.

If the probable relative error is to be reduced to the neighborhood of 5 per cent, the number of tubes must be at least 1000.

An examination of the total number of fertile tubes, without regard to the sets in which they occur, increases the probable error but slightly.

---

<sup>12</sup>loc. cit., p. 366.



## NEW AND RARE NORTH AMERICAN COLLEMBOLA

HARLOW B. MILLS<sup>1</sup>

*From the Department of Zoology, Iowa State College*

Accepted for publication January 22, 1932

Through the kindness of Mr. A. R. Rolfs of Yakima, Washington, there has come to the author's hands an excellent collection of Collembola representative of that region of the United States. The immediate purpose of this paper is to describe the heretofore unnamed species represented in this collection before the publication of the complete list for the state of Washington. Descriptions are included, also, of a number of forms from other parts of the country.

In addition, two species, *Architomocerura crassicauda* Denis and *Entomobrya triangularis* Schött, are discussed because of the apparent rarity of the former, and the incomplete original description of the latter.

The species treated in this paper are,

*Willemia denisi* n. sp.

*Entomobrya nigriceps* n. sp.

*Tullbergia iowensis* n. sp.

*Entomobrya triangularis* Schött

*Architomocerura crassicauda* Denis

*Pseudosinella rolfsi* n. sp.

*Entomobrya intonsa* n. sp.

*Salina decorata* n. sp.

*Willemia denisi* n. sp.

### Figures 1-4

Yellowish white, eyes absent. Furcula absent. Post-antennal organ (Fig. 2) with four tubercles. Mandibles two-toothed at the tip (Fig. 4), with a small molar surface bearing about four ridges, the right one with a sharp, oblique, inward pointing tooth at the base of the molar surface which fits into a shallow fossa on the left mandible. Antennae slightly shorter than the head, the proportions of the segments about as 17:21:21:19. Third antennal segment organ (Fig. 3) with two slightly capitate sense rods, guarded by two dorsal and three ventral setae. Fourth antennal segment with five large sense bulbs as in figure 3, and an apical, retractile, sense knob. Unguis without teeth (Fig. 1). Unguiculus about half the length of the unguis, acuminate, with an inner basal lamella. Clothing of a few short, slightly curving setae, longer on the fifth and sixth abdominal segment. One irregular transverse row of bristles on the femora and two rows on the tibiotarsi. A long latro-distal bristle extends on either side of the unguis. Anal horns absent. Length 1 mm.

Two specimens of this species were taken beneath moist boards near water at Yakima, Washington, March 23, 1931, by A. R. Rolfs.

<sup>1</sup>The author again wishes to express his thanks to Dr. J. W. Folsom of Tallulah, Louisiana, for the aid so freely given in the preparation of this paper.

It is contrary to good taxonomic practice to describe these minute insects from so few specimens. I do not hesitate in this case, however, for the species is evidently distinct. Because of lack of material it was impossible to investigate the organ of the third antennal segment completely, and some of the minute detail may have been overlooked.

The discovery of this species is of considerable interest as the genus is known only from the European *Willenia anophthalma* Börner and its subspecies *inermis* Börner. It approaches *W. anophthalma inermis* in the shape of the postantennal organ and in the absence of anal spines, but the sense organs of the fourth antennal segment are very different.

I take pleasure in naming this species for J. R. Denis, of the Laboratoire Arago, Banyuls sur Mer, France, who has kindly compared specimens of the American species with European forms for me.

The key following will aid in the identification of species in this genus.

1. Sense organs of the fourth antennal segment larger and bulbous. Postantennal organ of four tubercles. Anal horns absent. Washington, U. S. A. .... *denisi* n. sp.  
     Sense organs of the fourth antennal segment sub-cylindrical clubs. .... 2
2. Anal horns present. Postantennal organ of five or six tubercles. Europe ..... *anophthalma* Börner  
     Anal horns absent. Postantennal organ of four tubercles. Europe ..... *anophthalma inermis* Börner

*Tullbergia iowensis* n. sp.

Figures 5-8

Color entirely white. Slender, about six times as long as wide. Postnatal organ (Fig. 8) large, obovate, containing from 30 to 40 tubercles arranged in two rows. One poorly developed pseudocellus on each antennal base, one on each side of the base of the head, and one on each side of the mesonotum, metanotum, and first four abdominal segments. Antennae shorter than the head, the apical segments with about five enlarged olfactory setae each. Organ of the third antennal segment (Fig. 5) with an inner papilla, two converging sense clubs, two sense rods hidden behind an integumental fold, and three guard setae. Unguis (Fig. 7) broad, curving from the base, unarmed. Unguiculus represented by a sharp tubercle. Two anal horns, a little shorter than the hind unguis, moderately developed, strongly curved, on small noncontiguous papillae. Integument finely tuberculate. Clothing of rather slender hairs of medium length, becoming longer on the sixth abdominal segment. Two heavy spines (Fig. 6) on the posterior border of the fifth abdominal segment. Length 0.6 mm.

Taken rather commonly from leaf mould by means of a modified Berlese apparatus.

Iowa: Columbus Junction, December 25, 1931, L. Weber.

Jefferson, December 5, 1931, G. C. Decker.

Ames, November and December, 1931.

Louisiana: Tallulah, July 5, 1930.

Texas: College Station, January 23, 1931.

In specimens from Texas the postantennal organs were more elongate-oval than is shown in the figure, and contained the largest number of tubercles notes. The heavy bristles on the posterior border of the fifth abdominal segment were also more pronounced.

The only recorded representatives from North America of the genus *Tullbergia* Lubbock are *T. collis* Bacon of California and the variety *T. collis mexicana* Handschin of Mexico, together with published references to the genus from Iowa and New York.

The Nearctic species thus far described may be identified by the following key:

Length 0.6 mm. Thirty to forty tubercles in each postantennal organ, arranged in two rows. Anal horns shorter than the hind unguis. Eastern United States.....*iowensis* n. sp.

Length. 1.5 mm. Sixty to seventy tubercles in each postantennal organ, arranged in four irregular rows. Anal horns as long as the hind unguis. Western United States and Mexico.....*collis* Bacon

### *Architomocerura crassicauda* Denis

#### Figures 9-12

Color entirely white. Eyes (Fig. 9) six on each side, on dense blue eyepatches. Antennae longer than head. First two segments simple, sub-cylindrical, the last two sub-elliptical and annulated, of the proportions 12:15:34:44. Third and fourth abdominal segments nearly equal. Furcula reaching to the ventral tube, only slightly tapering apically. Proportions of the manubrium to the dentes to the mucro as 3:6:1. Mucro (Fig. 10) bilamellate, with an apical and subapical tooth and a smaller basal tooth on the outer lamella. Tibiotarsus with a partial apical segment. Unguis (Fig. 12) untoothed, slightly curved apically. Unguiculus broadly lanceolate, two-thirds the length of the unguis. Tenent hair not present. Body covering of three kinds: short, curving, reclinate setae over the whole body; large, smooth, straight bristles occasionally over the body, becoming more abundant posteriorly; and long, evident bothriotricha from the third abdominal segment back. Antennae densely covered with short, curving hairs. Length 0.75 mm.

The above description is from a single specimen taken under rotting wood near a river at Yakima, Washington, April 19, 1931, by A. R. Rolfs. The discovery of this species in North America is of considerable interest. It agrees well with the description and figures of Denis<sup>2</sup>. The specimen at hand does not show, however, the violet on the antennae which is present in the type material, and the postantennal organ is not visible. The specimen is likely a young one.

### *Entomobrya intonsa* n. sp.

#### Figures 13-15

Ground color gray to yellow, with dark blue pigment. Antennae violet but for bases of first and second segments. Head with light wash of

<sup>2</sup>Mitt. Zool. Staat. und Zool. Mus. Hamburg, 44:219-221, 1931.

PLATE I  
DESCRIPTION OF FIGURES

*Willemia denisi* n. sp.

- Fig. 1. Left middle foot.
- Fig. 2. Postantennal organ.
- Fig. 3. Left antennal tip.
- Fig. 4. Left mandible.

*Tullbergia iowensis* n. sp.

- Fig. 5. Sense organ, right antennal segment III.
- Fig. 6. Left spine, abdominal segment V.
- Fig. 7. Left hind foot.
- Fig. 8. Postantennal organ.

*Architomocerura crassicauda* Denis

- Fig. 9. Left eyepatch.
- Fig. 10. Mucrones.
- Fig. 11. Side view.
- Fig. 12. Right hind foot.



## PLATE I



H. B. Mill., del.

blue, becoming more dense posteriorly on the cheeks. Eyepatches black, irregular in outline. Prothorax faintly tinged with blue laterally. Meso- and metanotum broadly margined on the first two abdominal segments. Third segment with a broad, oblique, lateral spot which sometimes originates ventro-laterally on the second segment. Fourth segment with a broad, irregular, transverse median band, and a more narrow one just in front of the posterior border. Lateral sclerite of fourth segment variable in pigmentation, but the upper margin of the pigmentation typically scalloped. Third paratergite with some pigment. Fifth segment with a posterior band. Sixth segment with the posterior half pigmented. Coxae irregularly marked, generally as in the figure. Legs washed with blue which is most dense at the apex of the femora. Eyes (Fig. 15), eight on each side, packed closely together on an irregular eyespot; the two inner proximal eyes reduced. Antennae nearly three times the length of the head, relative proportions of the segments as 24:33:28:32 in an average of eleven specimens. Fourth segment feebly annulate. Fourth abdominal segment six to nine times the length of the third. Unguis straight, bearing one outer tooth, a pair of small pseudonychia, and three pairs of inner teeth, the first two pairs rather large, and the third pair small. Unguiculus lanceolate, unarmed. Tenent hair large, with a large, triangular, distal knob; shorter than the unguis. The slender marked bristle opposite the tenent hair variable in length, sometimes extending beyond the tip of the unguiculus. Manubrium five-sixths the length of the dentes. Dentes with dorsal corrugations ending about two and one-half times the length of the mucro from its tip. Mucro (Fig. 14) much as usual, the teeth rather short and heavy. Head and body covered with fringed, clavate hairs and short reclinate setae. First three antennal segments with long sensory setae, and the usual covering of short, curved hairs. Long, slender setae on the coxae, femora, and on the external surface of the tibiotarsi. Manubrium with long pinnate hairs dorsally, and short finer ones ventrally. Dentes with the usual pinnate hairs. Maximum length 2.8 mm.

In all of the specimens examined, the tibiotarsi showed evidence of weakness, perhaps a false joint, about a fourth of its length back from the tip.

As a variation, there may be slight evidences of pigment dorso-laterally on the disks of the meso- and metanotum.

This species bears a resemblance to the European *Entomobrya pulchella* Ridley. It differs from descriptions of *pulchella* chiefly in the color pattern and the denticulation of the claws.

Taken uncommonly under rotting wood at the following locations in Texas:

College Station, November 15, 1931.

Milano, April 15, 1931, V. A. Little.

Huntsville, June 26, 1931.

Clifton, July 29, 1931, V. A. Little.

### *Entomobrya nigriceps* n. sp.

#### Figures 16-19

Body gray-yellow, pigment purple to black. Antennae colorless but for the basal third of the first segment, which is dense blue-black. Head

deep blue-black with irregular lighter areas. Pigment of the body forming two irregular lines on each side. Prothorax pigmented dorsally. Mesothorax margined laterally and with a dorso-lateral line on each side. Metathorax and first three abdominal segments irregularly continuing the markings of the mesothorax. Fourth abdominal segment pigmented laterally along the suture which divides the dorsal from the lateral sclerite, the pigment extending dorsally near the middle to a dark spot from which a bothriotrix extends, also forming a transverse band just in front of the posterior margin. Lateral sclerites of the fourth abdominal segment lightly pigmented. Paratergite of the third segment with an elongate dark blotch. Fifth segment with a dense lateral spot. Sixth segment uncolored. Furcula pigmented only on the coxal piece of the manubrium. Legs with an apical band on the femora, which, together with the coxae, are washed lightly with blue on the two posterior pairs of legs. Body covered with long, fringed, capitate hairs which become longer and not markedly capitate on the fourth abdominal segment, and shorter, reclinate hairs. The capitate hairs extend onto the first antennal segment and are replaced on the second and third segments by more slender, pointed sensory setae; the fourth segment being devoid of long sensory hairs. Eyes (Fig. 19), eight on each side, the two inner proximal eyes somewhat reduced. Antennae long, three times the length of the head. An average of six specimens gave proportions of the segments as follows, 33:44:41:47. Fourth abdominal segment about six times the length of the third. Unguis (Fig. 17) straight, rather narrow, with a pair of external teeth one-fourth the unguis length from its base and three pairs of teeth on the inner margin. Unguiculus slender and pointed, reaching to a point midway between the first and second pairs of inner teeth of the unguis. Tenent hair a little shorter than the unguis. Furcula reaching the ventral tube. Manubrium three-fourths the length of the dentes. Heavy corrugations of the dorsal dental surface ending about twice the mucronal length from the tip of the dens; minute crenulations continuing on to the base of the mucro, which is of normal shape (Fig. 16). Length 3.2 mm.

This large, distinct species appears to be a rather uncommon resident of the leaf mould, where it was taken in association with *Orchesella ainsliei* Folsom. It is rather quiet in its actions, often remaining entirely motionless for some time when it fancies itself hidden behind a blade of grass. In spite of this habit it is extremely hard to catch, for it is a strong jumper and when disturbed may leap several times in succession.

Texas: College Station, March 12, 1931.

Bryan, May 5, 1931, Esther B. Mills.

Huntsville, June 26, 1931.

Tyler County, June 27, 1931.

Milano, July 1, 1931.

### *Entomobrya triangularis* Schött

Ground color light yellow, pigmentation purple. Apices of the first three antennal segments and the whole of the fourth colored, the latter but lightly. Pigment on the cheeks extending onto the sides of the pronotum. Mesonotum bordered anteriorly and laterally with pigment; metanotum with a broad dorsal area colored, and a continuation of the lateral lines

## PLATE II

*Entomobrya intonsa* n. sp.

- Fig. 13. Side view.  
Fig. 14. Right mucro.  
Fig. 15. Left eyepatch.

*Entomobrya nigriceps* n. sp.

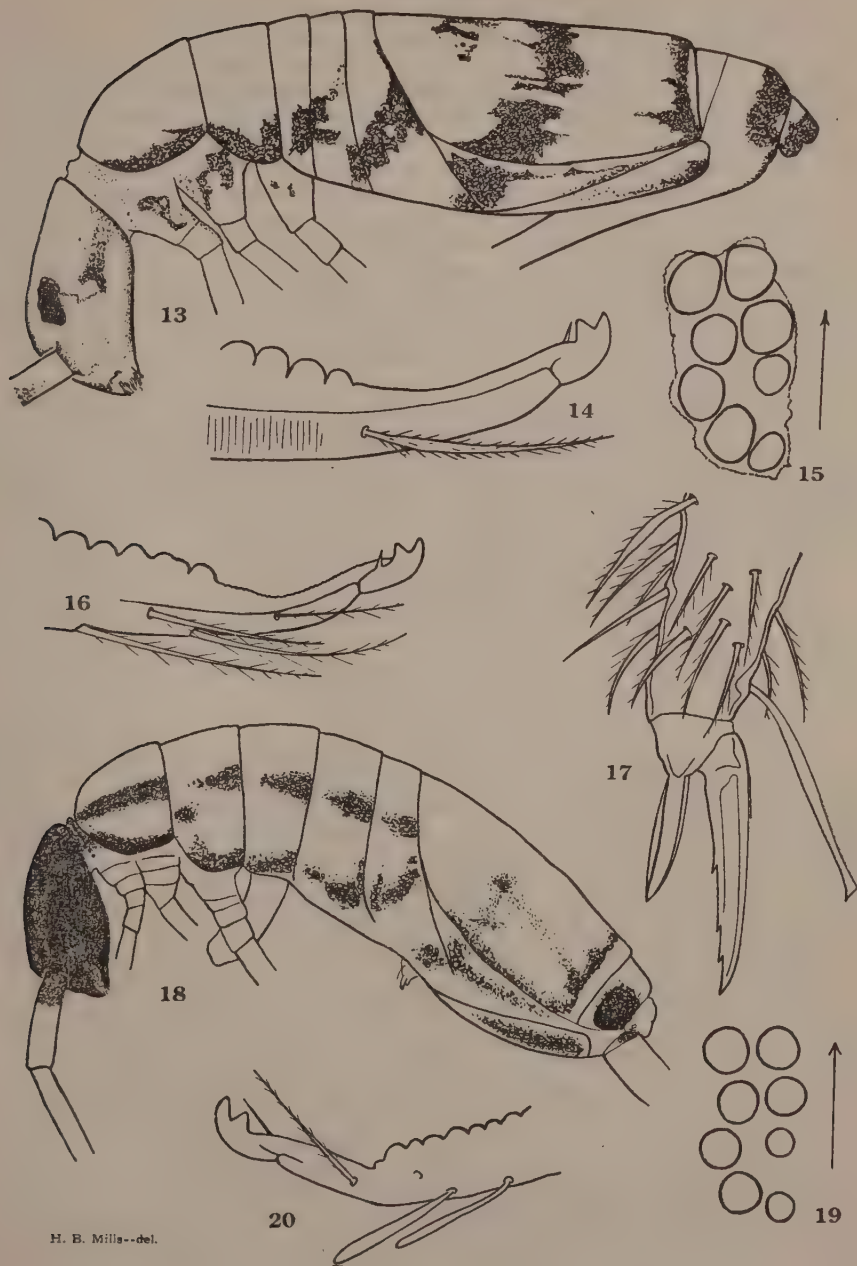
- Fig. 16. Left mucro.  
Fig. 17. Right hind foot.  
Fig. 18. Side view.  
Fig. 19. Left eyepatch.

*Pseudosinella rolfsi* n. sp.

- Fig. 20. Right mucro.



PLATE II



of the mesonotum. First abdominal segment lightly pigmented laterally; second segment colorless; third segment entirely colored but for the posterior lateral portions, the dorsal pigment extending slightly onto the anterior mid-dorsal portions of the fourth segment; fourth segment with a broad transverse median band becoming quite narrow across the dorsum; fifth segment with the posterior two-thirds colored; sixth segment without pigment. Apices of the hind femora with a band of light blue. Antennae one and one-half times the length of the head. Eyes, eight on each side of the head, the two inner proximal ones in each eyepatch smaller. Unguis rather straight, with three pairs of teeth on the inner lamellae, and a pair of pseudonychia. Unguiculus two-thirds the length of the unguis, rather broadly lanceolate, untoothed. Tenent hair slender, subequal to the unguis. Manubrium about two-thirds the length of the dentes. Corrugations of the dentes continuing very minutely to the bases of the mucrones, which are of the normal shape. Fourth abdominal segment four to five times the length of the third. Length 2 mm.

The specimens at hand agree very well with the description and figure of Schött<sup>3</sup>, who described the species from a single specimen taken in California. In his discussion of the color pattern, however, he states that the sixth abdominal segment is colored instead of the fifth (the figure showing the opposite to be true), and he apparently missed the distal pair of teeth on the inner lamellae of the unguis.

Washington: Tampico, March 1, 1931, A. R. Rolfs.

Puyallup, April 25, 1931, A. R. Rolfs.

Tacoma, April 26, 1931, A. R. Rolfs.

*Pseudosinella rolfsi* n. sp.

Figures 20, 29

Color entirely white. Eyes absent. Antennae about one and one-half times the length of the head, the first segment slightly more than half the length of the second, which is subequal to the third; fourth segment more than twice the length of the third and elongate-elliptical. Mesonotum extending completely over the pronotum. Fourth abdominal segment four times the length of the third. Unguis broad, slightly curving at the tip, bearing a single small tooth beyond the middle of the inner margin and an extremely large lamellated tooth arising from the posterior lamella at the base (Fig. 29). Unguiculus slender, reaching as far as the single small inner tooth of the unguis, and bearing an obliquely truncate lamella for two-thirds of its length externally. Tenent hair very heavy, with a large distal swelling and a basal flattening which seems to be characteristic; slightly shorter than the unguis. Manubrium to the dentes about as 27:42. Dental corrugations strong, ending abruptly about the length of the mucro from the dental tip. Mucro (Fig. 20) of the usual type, the basal spine long. Body covered with fringed clavate hairs. The specimens were killed in hot alcohol, and the body scales are not apparent. Dentes scaled ventrally and with lateral pinnate setae. Length 1.5 mm.

This species is related to the European *Pseudosinella petterseni* Börner, and *P. violenta* Folsom and *P. folsomi* Denis of North America.

<sup>3</sup>Proc. Calif. Acad. Sci., 2nd series, 6:182-183, 1896.

It agrees with the genus in the presence of the large "wing tooth" of the unguis, the reduction in pigment and in number of eyes, but is distinct in the size of the tenent hair which is large even for the normal *Lepidocyrtus* forms.

This species, I take great pleasure in naming for its discoverer, Mr. A. R. Rolfs, who collected the six co-types from beneath stones at Yakima, Washington, March 22-24, 1931.

*Salina decorata* n. sp.

Figures 21-28

Body color gray to yellow. Irregular lateral blue-brown blotches on head. Small blue spot on vertex and a line connecting the eyespots through the antennal bases. Lateral blue margins on the meso- and metanotum, and first abdominal segment, continuing in company with a ventro-lateral blue line on the second and third abdominal segments. Another indefinite blue line begins dorso-laterally on the third abdominal segment and continues on to the fourth, where it breaks into a number of blue blotches. Fifth and sixth segments blue laterally. A faint, brown, median longitudinal line on vertex, extending onto the body, bifurcating on the mesonotum, and continuing as two brown dorso-lateral lines as far as the middle of the fourth abdominal segment, where each line ends in a blue-brown spot. A median brown line begins where these lines end, continuing to the posterior margin of the fourth segment. The brown dorso-lateral lines are usually interrupted on the third abdominal segment, and the single posterior median brown line of the fourth is often more red-orange than brown. Antennae light blue, the first two segments light basally with dark distal rings. Legs washed with blue, which deepens to apical bands on the femora; blue freckles on the femora and in darker specimens on the tibiotarsi also. Furcula colorless or slightly washed with blue. Eyes (Fig. 24), eight on each side, the two inner proximal ones smaller, on deep brown eyepatches. Antennae longer than the body (as 4:3), the relative lengths of the segments in one specimen as 21:37:33:36. Third abdominal segment to the fourth about as 4:45. Unguis (Fig. 23) with two pairs of inner teeth. Unguiculus broad, with two ribs: obliquely truncate distally. Tenent hair half again as long as the unguis, minutely pubescent. Manubrium slightly more than two-thirds the length of the dentes. Mucrones (Figs. 25-28) normally tridentate, varying nearly to a bidentate form. Dentes with the usual bladder-like structure apically. Body covered with many short, reclinate setae. The available material is rubbed, so the large fringed, clavate hairs are largely absent. Maximum length 2.1 mm.

The specimens from Texas were taken running about on the bark of pine logs after a rain. They were very active.

Florida: Gainesville, March 12, 1925, T. H. Hubbell.

Texas: Tyler County, June 27, 1931, H. B. Mills, V. A. Little and S. R. Warner.

This species resembles the second form of *Salina trilobata* described by Schött from Mexico<sup>4</sup>, and also *S. tricolor* (Handschin) of India. From

---

<sup>4</sup>Proc. Calif. Acad. Sci., 2nd series, 6:175-178, 1896.

## PLATE III

*Salina decorata* n. sp.

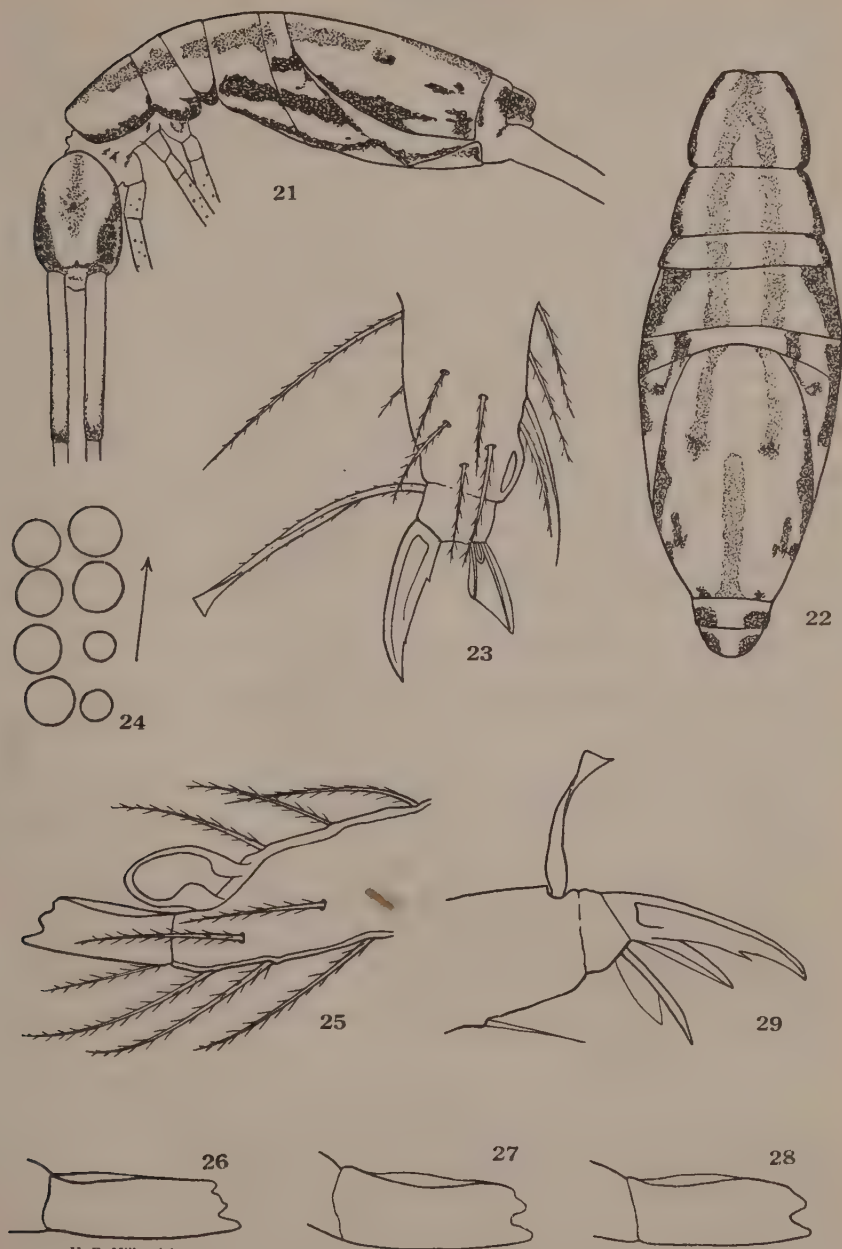
- Fig. 21. Side view.  
Fig. 22. Dorsal view.  
Fig. 23. Right middle foot.  
Fig. 24. Left eyes.  
Fig. 25. Right mucro.  
Figs. 26-28. Variations in mucrones.

*Pseudosinella rolfsi* n. sp.

- Fig. 29. Left hind foot.



## PLATE III



H. B. Mills--del.



## NECTARIES OF CAPSICUM

J. N. MARTIN, A. T. ERWIN AND C. C. LOUNSBERRY

*From Plant Morphology Laboratory, Botany Department, Iowa State College*

Accepted for publication March 18, 1932

The purpose of this article is to report the presence and describe the structure and functioning of the nectaries in some of the varieties in the genus, *Capsicum*. The secretion of nectar has not been considered characteristic of this genus of the Solanaceae. The authors found no account of nectaries within the genus *Capsicum*. The failure of nectaries in this genus to attract the attention of biologists is ascribable to the absence of conspicuous accumulations of nectar and to the lack of special interest in the flowers on the part of bees. The pungency of the capsaicin present in the nectar is probably responsible for the indifference of bees toward the flowers<sup>1</sup>.

### LITERATURE

The reports on other genera of the Solanaceae indicate considerable diversity in respect to nectaries.

Bonner (1) states that, in most Solanaceae, the sugar is distributed through the parenchyma of the ovary with no special place of emission. In *Lycium barbarum*, Bonnier says the nectariferous tissue is localized in the bases of the carpels. This is in accord with Müller's (4) statement that honey is secreted in large quantities by the ovary in *Lycium barbarum*.

According to Knuth (3), who refers to nectaries in ten genera of the Solanaceae, the nectar is secreted below the ovary in the genera, *Physalis*, *Atropa* and *Mandragora*; by the base of the ovary in the genera, *Lycium*, *Nicandra*, *Hyoscyamus*, *Nicotiana*, and *Datura*; by an annular disk below the ovary in the genus *Scopolia*; and by a swelling at the base of the ovary in the genus, *Physochlaina*.

In the flowers of *Datura stramonium*, Knuth (3) describes five tubular passages at the base of the corolla. These tubular passages are the grooves between the filaments which are adnate to corolla tube by their narrow posterior margins. Through the expansion of the inner margins of the filaments the grooves are roofed over and become tubular. Knuth refers to a statement by Kerner that similar grooves are present in the corolla of *Physalis Alkekengi*. Knuth describes pairs of capitate projections between the corolla lobes in *Solanum Dulcamara*. These he regarded as nectaries.

### FLORAL STRUCTURES RELATED TO THE NECTARIES

The corolla in the genus *Capsicum* is gamoptelous. The basal portion is a short tube a few millimeters in length and more or less cylindrical in shape. From the top of the corolla tube the petals flare outward, forming corollas

---

<sup>1</sup>The most nectar found was in the Tobasco and Cayenne groups. Among those varieties with nectar most noticeable were Chili, Anaheim Chili, Short Thick Cayenne, Long Slender Cayenne, Tobasco, Japanese Miniature, Japanese Ornamental, and Cerise.

ranging from bell- to wheel-shaped in the different types. The stamens alternate in position with the lobes of the petals. The bases of the filaments are adnate to corolla tube. The separation of filaments from corolla tube occurs a short distance below the rim of the tube, resulting in the presence of narrow spaces between backs of filaments and top portion of tube. The corolla-filament tube is comparatively thick and closely jackets the ovary. Between the filaments and near the top of the corolla tube occur pairs of projections (Plate I, fig. 4). These projections arise from the inside and near the rim of the corolla tube. In some varieties they are so prominent as to suggest aborted stamens. These projections apparently correspond to those in *Solanum Dulcamara*, which have been described by Knuth (3) and regarded by him as true nectaries.

Between the members of each pair of projections is a deep but narrow groove traversing almost the entire length of corolla tube (Plate I, fig. 4). The grooves are deepest at their upper end, reaching almost to the mid-vein of the petals (Plate I, fig. 3, and Plate II, fig. 2). They are similar to the grooves referred to in *Physalis Alkekengi*. When secreting is in progress these grooves serve as tubes, and their openings at their upper ends are the pores through which much of the nectar reaches the surface of the petals (Plate I, figs. 1 and 4.).

Also between the filaments of the stamens and corolla tube occur duct-like openings where nectar accumulates (Plate I, fig. 2). These ducts, one back of each filament, are formed through the gradual separation of filaments from corolla tube. The separation begins as a slit-like opening between back of filament and corolla tube. This slit-like opening widens and gradually extends in a semi-circle as the top of tube is approached until it cuts through to inner surface of tube, thus making the separation of filament and corolla complete (Plate I, fig. 2). These duct-like spaces between filaments and corolla both secrete and conduct nectar.

#### CHEMICAL STUDY OF THE NECTAR

Drops of the secretion were pressed from flowers onto a slide and tested for sugar. With Fehlings' and Fluckiger's reagents, abundant cuprous oxide appeared after heating. With phenyl hydrazine hydrochloride, osazone crystals formed in abundance (Plate I, fig. 5). The osazone crystals formed during and immediately after the cooling of the solution. They were yellow, slender, sharp pointed crystals, having the characteristics of the osazones of fructose and dextrose (Plate I, figs. 7 and 8). However, the test with Fluckiger's reagent for fructose was negative. The drops of secretion also contained much capsaicin which was detected by its pungency.

When tests for sugar were applied to the corolla tube cuprous oxide crystals and osazones formed in abundance, not only on the surface but within the tissues of the corolla and filaments of the stamens (Plate I, fig. 6). The vascular bundles in basal portions of filaments and petals were crowded with osazones. Either the sugar becomes generally distributed by diffusion from places of formation or its formation is a function common to most of the cells in the bases of the petals and filaments.

The thickness of the tube formed by fusion of filaments and corolla tube is greatest just below the rim of the tube. In this region the grooves are deepest, and the paired projections are present. This portion of the tube, on account of its greater thickness, is cushion-like in appearance. It



was in the tissues of this region that the greatest abundance of crystals occurred (Plate I, fig. 6). There was only slight evidence of sugar at the base of the corolla tube and in the corolla above the tube. In the filaments of the stamens the osazones were abundant only in their bases, only a few occurring above the level of the rim of the corolla tube.

In cross and lengthwise sections of the entire flower subjected to the osazone test, the greatest display of sugar occurred on the inner surface of the corolla tube in the region of grooves. Here the crystals were extremely large and aggregated into mammoth clusters. In the tissues of the corolla there were many clusters of small crystals. In the ovary wall the crystals were few. In the placentas the crystals were about half as numerous as in the tissues of the corolla. They, too, were small and aggregated into small culsters. No formation of mucilage was observed in any part of the ovary. There was no indication that the ovary functions as a nectary as has been reported in *Lycium barbarum*.

#### THE SECRETING MECHANISM

Accompanying the appearance of the secretion, certain cells in the thick, cushion-like region of the corolla-filament tube undergo changes that suggest they have a special function in the secretory process. The most marked of these modifications is the formation of a thick layer of mucilage in the inner epidermis of the cushion-like region of the tube.

Previous to the opening of the flower and to the appearance of nectar, the cells of the inner epidermis of the tube have dense protoplasm with much starch in both inner and outer regions (Plate II, fig. 1). The outer cell wall is comparatively thick. It consists of three rather distinct layers (Plate II, fig. 1). The outermost layer is cuticle, giving a definite reaction for cutin with micro-chemical tests. The middle layer reacts with cellulose tests, swelling and becoming blue almost immediately when chlor-zinc-iodide is applied. The inner layer is cellulose, but much more resistant to reagents than the middle layer.

During the process of secreting the middle layer swells, becomes mucilaginous and pushes up the cuticle, forming large blister-like elevations (Plate I, fig. 3 and Plate II, fig. 2). The cuticle is finally ruptured and the secretion oozes out to the surface, through the layer of mucilage, causing the inner surface of the cushion-like region of the corolla-filament tube to have a dull, glistening appearance. While the modifications are taking place in their outer wall, the starch in the protoplasm of the epidermal cells disappears, a fact suggesting that the starch is utilized in the formation of the mucilage and nectar.

The most general involvement of epidermal cells in the formation of the mucilaginous secretion is displayed in and around the grooves. Through the concerted action of all the epidermal cells in this region, there is a uniform accumulation of mucilage and the cuticle is elevated as one large blister (Plate I, fig. 3, and Plate II, fig. 2). The only epidermal cells not displaying modifications in this region are those in the bottom portion of the grooves. This feature permits the bottom portion of the grooves to remain open and serve as tubes when the upper portions of the grooves are choked with the expansion of the epidermal cells through the swelling of their walls. The upper ends of these tubes are the pores noticeable on the petals at the top of the corolla tube. The secretion oozing from sides of

the grooves and from nearby regions of the inner epidermis of the corolla-filament tube accumulates in the bottom of the grooves and they afford the outlet to the surface of the petals.

Through the displacement of the middle layer by the swollen mass of mucilage, the cell walls are much distorted and thickness greatly increased. The elevating and stretching of the cuticle results in its destruction. The inner layer of wall shows no noticeable modifications. Its outer border, however, is not distinct from the mucilage, and thus there is the possibility that its outer portion may be included in the process of gelatinization. The density of mucilage decreases towards the exterior, its percentage of water being high in the region of the cuticle.

The cells of the outer epidermis of the corolla tube, as compared with those of the inner epidermis, are smaller, the contents less dense, and the outer wall is not so thick. At places, but less generally, the outer epidermal cells behave as the inner. The middle layer of their outer wall swells, thus forming the blister-like elevations and rupturing the cuticle (Plate I, fig. 3). The location of these areas is commonly opposite a groove and thus near the main veins of the petals, but they occur anywhere on the outer surface of the tube. Whether or not nectar appears in appreciable amounts on the outer surface of the corolla tube was not determined.

In the duct-like spaces between the backs of the filaments and the corolla tube, the epidermal cells of both filaments and corolla appear almost as active as those in and around the grooves. The epidermal cells, the entire way around the filaments just above the place where the filaments become free from the corolla tube, are mucilaginous and apparently secreting. In the tests the vascular bundles in this region of filaments showed an abundance of sugar. The nectar oozing from these areas is the liquid which often fills the spaces between filaments and the rim of the corolla tube.

The sub-epidermal and all cells within the corolla-filament tube, excepting vascular tissue, have thin cellulose walls and thin peripheral layers of protoplasm. They contain some starch, but much less than epidermal cells. As previously stated, at the time nectar is being secreted, both cuprous oxide and osazones resulting from the respective tests appear scattered throughout the entire thickened or cushion-like region of the corolla-filament tube with greatest concentration in the vascular bundles of both the corolla and filaments (Plate I, fig. 6).

Occasionally masses of mucilage were observed here and there in the parenchyma tissue of the corolla-filament tube. They usually occupied the spaces of a number of cells. A study of their formation revealed a dissolving of cell walls and fusion of cell contents. In the sugar tests there was no evidence that these internal mucilaginous regions contained more sugar than surrounding tissue. They were found only in a few flowers, and were considered of no special significance.

The secreting mechanism in the genus *Capsicum*, as shown by the forms included in this study, is not a distinct morphological structure, as typical nectar glands are. It consists of the corolla tube and adnate basal portion of the filaments. The inner epidermis of the corolla-filament tube, because of its formation of much mucilage, participates most noticeably in the secretory process. The parenchyma of the corolla-filament tube apparently contributes some of the sugar in the nectar through the conversion of the starch it holds in storage, while it is probable that the vascular bundles

of the corolla-filament tube contribute sugar directly from the transportation stream.

#### SUMMARY AND DISCUSSION

A number of the forms in the genus *Capsicum* secrete a liquid which accumulates in noticeable drops on petals and in spaces between the backs of filaments and the corolla tube. Tests show that this liquid contains an abundance of dextrose, and may be regarded as nectar.

The nectar is secreted by the tube at the base of the corolla. This tube is the result of the fusion of the corolla tube and the bases of the filaments. On the inside of this tube and between the filaments are grooves that open as pores on the inner surface of the petals at about the level or a little above the rim of the corolla tube. These grooves function in secreting, collecting, and in conducting the nectar to the surface of the petals. Between the filaments and the corolla tube, the nectar often fills the space to the level of the rim of the tube.

The feature of the secreting process most noticeable is the accumulation of mucilage in the outer cell walls of the inner epidermis of the corolla-filament tube. This is manifest most in and around the grooves on the inner side of the tube and between the backs of the filaments and the corolla tube. The mucilage replaces the middle layer of the outer wall of the epidermal cells. Its accumulation elevates and destroys the cuticle. Through the layer of mucilage the secretion oozes to the surface.

There may be two sources of the sugar present in the nectar. Much of it may result from the conversion of the starch so generally present throughout the tissues of the corolla-filament tube. Evidences of this source of the sugar are the disappearance of the starch and the general distribution of sugar throughout these tissues at the time of the formation of the nectar. The other source suggested is the vascular bundles. The abundance of sugar in the vascular bundles of the corolla-filament tube at the time the nectar is appearing suggests that at least some of the sugar in the nectar may come directly from the transportation stream.

The apparent source of the mucilage in the epidermal walls is the middle layer. The thickness of the middle layer previous to the formation of the mucilage, its replacement by the mucilage, and the similarity of the middle layer and mucilage in reaction to cellulose tests are evidences of this relationship. However, there is the possibility that the middle layer may be, in part at least, a place for the accumulation of mucilage formed in the protoplasm or from materials transported from the protoplasm of the epidermal cells. Hanstein (3), who made an extensive study of the formation of slimes and resins in leaf buds, describes many cases in which the slime is in the middle layer of the cell wall. He concluded that in some cases the slime arose entirely from the middle layer, and in other cases partially from protoplasmic contents transported into the middle layer, where they are changed into cellulose and then into slime or directly into slime. He reported the transformation of the middle layer into slime in some of the epidermal walls in the leaf buds of the genera *Datura* and *Nicotiana* of the Solanaceae.

The layer of mucilage decreases rapidly in density towards its outer border, where its fluidity approaches that of water. The nectar is the liquefied mucilage plus sugar and also other elements incidentally present.



The mucilage apparently is the mechanism responsible for the exudation of the nectar. What appears to take place is that the dense inner region of the layer of mucilage imbibes the solutions from the tissues beneath while the outer region of the mucilage, through liquefaction and resulting loss of imbibition, permits the solutions to flow away.

## LITERATURE CITED

1. BONNIER, GASTON  
1879. *Les Nectaries*. Paris.
2. HANSTEIN, JOHANNES  
1868. Ueber die Organe der Harz- und Schleim-Absonderung in den Laubknospen. *Bot. Zeit.*, 26:688-783.
3. KNUTH, DR. PAUL  
1909. *Handbook of flower pollination* (Eng. trans.), 3:152-160. Oxford.
4. MÜLLER, PROFESSOR HERMANN  
1883. *The fertilization of flowers*, pp. 425-428. London.

## PLATE I

Fig. 1. Flowers of one of the varieties of the genus *Capsicum* with pores where drops of nectar are found indicated at *O*.

Fig. 2. Cross-section of corolla-filament tube and ovary. Section is through thick portion of tube. The duct-like spaces between filaments and corolla tube are indicated at *a* and the grooves of the tube at *b*.

Fig. 3. Cross-section of a portion of the corolla-filament tube showing at *u* the gelatinization of epidermal walls in and around grooves on inner surface of corolla-filament tube and also on outer surface of tube. At *v* is the vascular bundle immediately below the groove.

Fig. 4. Interior of corolla-filament tube showing at *a* the projections present on corolla tube between filaments and showing at *b* the grooves on the inner surface of tube.

Fig. 5. Osazones resulting from sugar test on drop of nectar from a *capsicum* flower.

Fig. 6. A portion of corolla-filament tube after being heated in phenyl hydrazine hydrochloride and sodium acetate. At *a* are two filaments with vascular bundles filled with osazones. At *b* is the thickened portion of corolla-filament tube where osazones are most abundant, especially in the parenchyma tissue of tube.

Fig. 7. Mammoth clusters of large osazone crystals that formed on surface of corolla-filament tube.

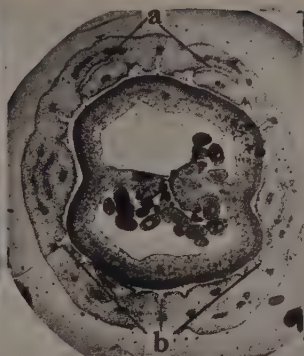
Fig. 8. Smaller clusters of osazones that form in the parenchyma of thickened portion of corolla-filament tube.



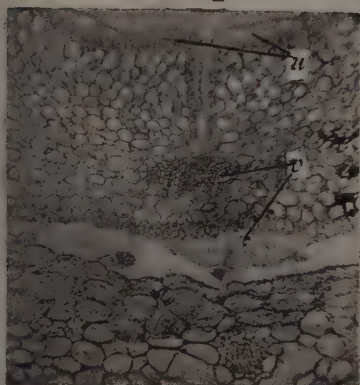
PLATE I



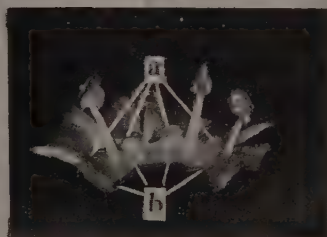
1



2



3



4



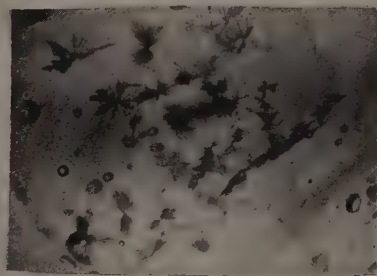
5



6



7



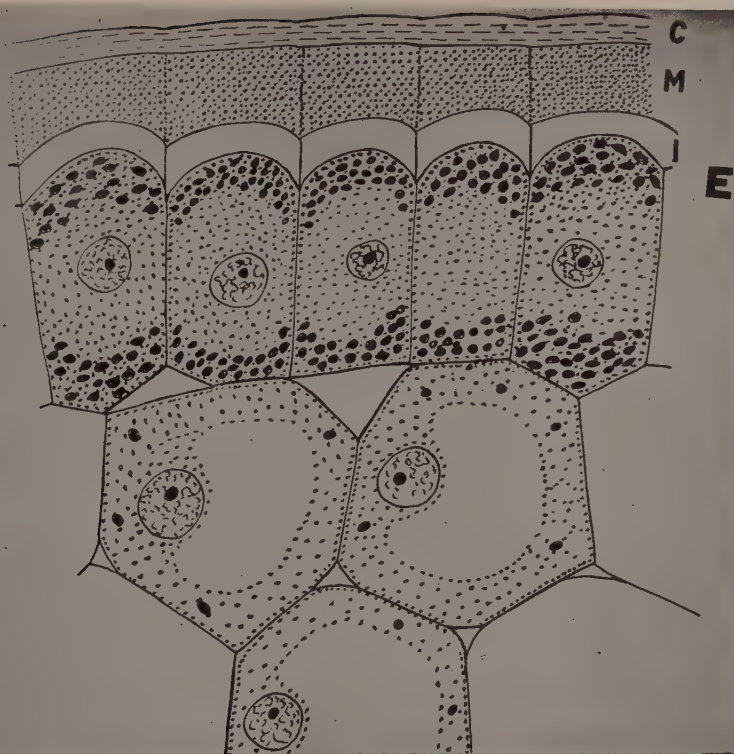
8

## PLATE II

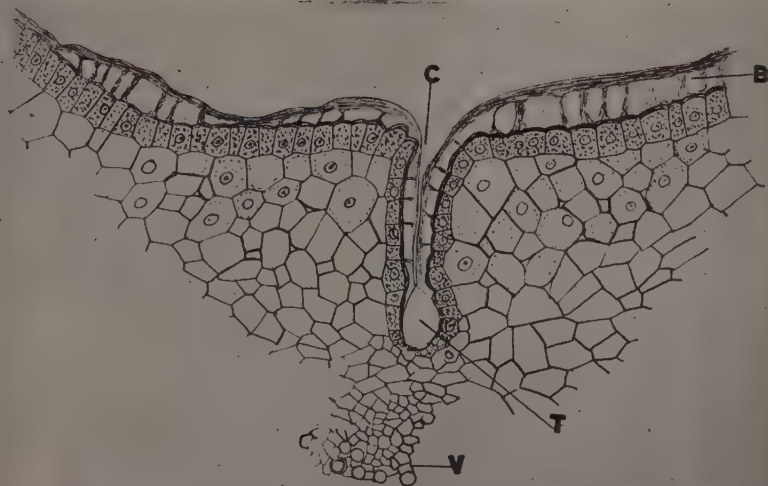
Fig. 1. A portion of a cross-section of corolla-filament tube showing inner epidermis *E* previous to the appearance of the mucilage in middle layer. Also three parenchyma cells below. Cuticle is indicated at *C*, middle layer at *M* and inner layer of wall at *I*.  
The dark bodies in protoplasm of cells represent starch.

Fig. 2. Cross-section of a portion of the corolla-filament tube at time secreting is in progress. *C*, groove closed for most of its depth by gelatinization of epidermal cell walls. *T*, bottom of groove remaining open and serving as a tube for conduction of nectar. *B*, layer of mucilage forming in middle layer of outer epidermal wall. *V*, vascular bundle.

PLATE II



1



2





# THE RELATIVE TOXICITY OF PYRIDINE AND NICOTINE IN THE GASEOUS CONDITION TO TRIBOLIUM CONFUSUM DUVAL<sup>1</sup>

CHARLES H. RICHARDSON AND LOUISE E. HAAS

*From the Department of Zoology and Entomology, Iowa State College*

Accepted for publication March 31, 1932

In order to compare the toxicities of a series of compounds to a living organism, one must first ascertain the concentration of each compound that permits the survival, in a certain time interval, of approximately one-half the population of the organism, 6, 21, 2. This is usually accomplished by making a number of experiments from the results of which toxicity curves may be plotted with concentration or time as the independent variable and the percentage of mortality as the dependent variable. From these curves, the median lethal concentrations, or 50 per cent points, may be calculated.

In an investigation of this kind, the question of the relation of the toxicity ratio to exposure time is of first importance. For example, the question may fairly be asked whether the relative toxicity of two compounds determined at five hours is the same as that determined at two hours (19). Obviously, if this ratio is altered with a change in time, it must be considered as a specific rather than a general attribute of the toxic compounds.

The investigation of the relative toxicity of pyridine and nicotine was undertaken for several reasons. First, it was desirable to answer, if possible, the above question. Furthermore, the relative toxicity of these compounds, the molecules of which have a certain structural relationship, are of interest on theoretical grounds. In addition, both compounds have been used to control insects, and nicotine is at present widely employed for this purpose. Accurate determinations of their toxicity in the gaseous condition to insects have not yet appeared in the literature.

The writers are indebted to Dr. R. M. Hixon, Department of Chemistry, Iowa State College, for suggestions concerning the design and operation of apparatus, and for samples of pure chemical compounds; and to Professor G. W. Snedecor, Department of Mathematics, for advice on statistical matters. To Dr. L. C. Craig, they acknowledge generous aid in several ways.

## HISTORICAL

In searching for fumigants, a number of investigators have experimented with pyridine and nicotine. Trillat and Legendre (22) made tests on insects with these compounds in 1909. McClintock, Hamilton and Lowe (14) found that nicotine was invariably more effective as a fumigant to several species of insects than was pyridine. Using nearly saturated atmospheres upon the oriental cockroach, *Periplaneta orientalis* (L), Holt (11) found that pyridine was lethal in 75 minutes, nicotine in 180 minutes.

---

<sup>1</sup>Journal Paper No. B49 of the Iowa Agricultural Experiment Station.

The concentration of the former compound, under these conditions, was probably 30 times that of nicotine. Moore (15) killed houseflies, *Musca domestica* L., in 400 minutes at 21°C. with 21.7 millionths of a gram molecule/liter of pyridine and 2.4 millionths of a gram molecule/liter of nicotine. Nicotine was therefore 9 times more toxic than pyridine on the molecular basis and 4.6 times more toxic when concentration was expressed as milligrams/liter. The criterion of toxicity was the death of 100 per cent of the insects used. According to Moore and Graham (16), the eggs of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), are killed by 1.8 millionths of a gram molecule/liter of nicotine, the exposure time being 15 hours. Tattersfield and Roberts (20) found pyridine to be moderately toxic to wireworms; death occurred at a concentration of 6 mg./liter and recovery at 4.7 mg./liter in 1,000 minutes, the temperature being 15°C. De Ong (5) gives some data on the toxicity of nicotine in gaseous form to aphids, which, however, are difficult to interpret in this connection. Neifert, et al (18), included pyridine among a large number of organic compounds which they tested on several species of grain-infesting insects. Pyridine was stated to be effective at 2.9 g./liter (0.83 molar per cent), the exposure time being 24 hours.

In the above studies, no determinations of the median lethal concentration were made. Jewson and Tattersfield (12), however, give this information for pyridine, the mite, *Tyroglyphus longior* Gervais, being the test organism. At a concentration of 5 mg./liter and a three hour exposure, 50 per cent of the mites survived. The temperature was 15° to 18° C. More recently, Craig (3) has studied the relative toxicity to *Tribolium confusum* of a group of nitrogen heterocyclic compounds which included pyridine and nicotine. Pyridine killed 50 per cent of the insects at a concentration of 34.6 mg./liter in 158 minutes, whereas nicotine in the same time required 0.031 mg./liter. The toxicity ratio, 1,116, is very materially larger than that obtained with our insects. The difference seems to depend, in part at least, upon the fact that the age limits and nutrition of the larvae were more closely controlled in our cultures than in those used by Craig.

#### MATERIALS

The insects used in this investigation were adults of the confused flour beetle, *Tribolium confusum*. Individuals of known age limits were obtained by removing the adults from the stock cultures in whole wheat and placing them in white flour, the food value of which had been increased by the addition of three per cent of brewer's yeast. Every twenty-four hours, the flour was sifted through a number 20 sieve (U. S. standard) to remove the beetles and then through sieve number 60 to remove the eggs. The eggs, which were obtained from the same lot of beetles for not longer than one week, were placed in coarsely ground whole wheat flour in wide culture dishes, where they hatched, and the larvae completed their development. The temperature was held at 25° C., the relative humidity at 60 to 70 per cent. Adults were used in the experiments when they reached an age of 20 days, and were in most cases discarded when more than 50 days old.

The compounds used were as follows: Pyridine (Merck) redistilled, boiling point 112°-113° C.; nicotine prepared from the hydrochloride according to the method of Harlan and Hixon (10). Another sample, analyz-

ing 99.32 per cent nicotine, was prepared by distillation from 98 per cent nicotine.

### THE APPARATUS

The apparatus was patterned in general after that of Neifert and Garrison (17), who were probably the first to describe the use of flow meters to measure the concentrations of gaseous insecticides. As nicotine is strongly absorbed by rubber (9), that portion of the apparatus traversed by the gas was constructed of Pyrex glass without rubber connections. (Fig. 1.)

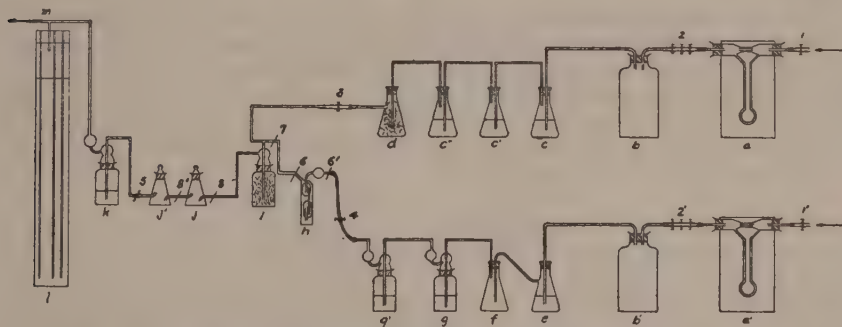


Fig. 1. The Apparatus: *a, a'* flow meters; *b, b'* air cushion bottles; *c, c', c''* dehydrators or humidifiers; *d* trap flask containing glass wool; *e* sodium hydroxide flask; *f* trap flask; *g, g'* dehydrators; *h* absorption bulb for toxic compound; *i* mixing flask; *j, j'* exposure flasks for insects; *k* absorption flask containing dilute sulphuric acid; *l* pressure regulator; *m* T-tube; 1, 1', 2, 2', 3, 4, 5, Hofmann clamps; 6, 6', 7, 8, 8' ground glass joints.

All glass joints, 6, 6', 7, 8, 8', were ground and polished until they were gas-tight. The air was drawn through the apparatus by means of a suction pump, its volume being measured in calibrated flow meters (4). The air which entered flow meter *a'* was first drawn through 20 per cent sodium hydroxide solution *e* to remove carbon dioxide, since nicotine readily absorbs it from the air, then through concentrated sulphuric acid scrubbers *g, g'* to extract the moisture. It then entered an absorption bulb of three compartments, *h*, where it was saturated with the compound under test. From here, it passed to a mixing flask *i*, where any desired concentration of the gas below saturation could be secured by dilution with air from flow meter *a*. The air flowing through *a* was dried in concentrated sulphuric acid or brought to the desired relative humidity in dilute sulphuric acid solutions or saturated sodium chloride solutions, *c* to *c''*, according to the demands of the experiment. After the desired concentration of toxic compound was obtained, the air was passed into the exposure flasks *j, j'* in which the insects were placed. Flask *k* contained dilute sulphuric acid to absorb the toxic compound from the air after it passed through the exposure flasks.

As the negative pressure produced by the water pump varied with the water pressure, over long periods of time, a pressure regulator *l* was inserted in the system to stabilize the rate of air flow. This regulator consisted of a large glass tube 95 cm. in length, filled with water, and fitted with a rubber stopper through which passed an arm of a T-tube *m*, and three glass



tubes of three cm. diameter. The glass tubes reached to the bottom of the large tube and were open at the top. The negative pressure at the suction pump was so adjusted that air was simultaneously drawn through the system and through the three long glass tubes. If only a single long tube is used in the regulator, the bubbling is so violent that the flow of air becomes irregular (4). The large empty bottles, *b*, *b'*, act as air cushions to reduce fluctuations in the flow meters due to bubbling in the pressure regulator and in other parts of the system. The air flow through the system was brought to the desired rate by means of Hofmann clamps 1, 1', 2 and 2'.

At the beginning of each experiment, the system was tested for leaks by closing clamps 1 and 1' before starting the suction pump. If, after a few minutes, no bubbles appeared in *k*, the system was air-tight.

The figures for the vapor concentration of the compounds in the air at 25° C. were obtained from the loss in weight of the compound after a known volume of air had passed through the absorption bulb. Table 1 shows the values obtained with this apparatus.

TABLE 1. *Vapor concentration of pyridine and nicotine at 25° C. and 742 mm. pressure*

Compound	Trials	Loss in weight grams	Air passed through compound liters	Concentration gram/liter
Nicotine .....	1	.0167	41.81	0.00040
" .....	2	.0154	36.02	.00043
	Av. ....			.00042
Pyridine .....	1	.0826	0.70	.1180
" .....	2	.1152	1.00	.1152
" .....	3	.1144	1.00	.1144
	Av. ....			.1159

The mean value for pyridine corresponds quite closely with that found by Craig (unpublished notes); the value for nicotine disagrees with that obtained by Harlan and Hixon (8), with a different type of apparatus, but agrees closely with that of Craig (unpublished notes) obtained under similar conditions. The difference observed is probably due to differences in the apparatus and to the presence of traces of impurities in our sample of nicotine.

#### METHOD OF EXPOSURE

After the Hofmann clamps were adjusted to give the proper rates of flow through the two flow meters, 15 or 20 minutes were generally required to bring the reading to a constant value and to fill the system with the gas-air mixture. Clamps 3, 4 and 5 were then closed, 50 insects were quickly dropped through a small funnel into each exposure flask, the glass stoppers were replaced, and the clamps were again opened. After the exposure was completed, the time was noted. Each sample of insects was removed to a small bottle containing whole wheat flour and covered with cheese-cloth. The beetles were then placed in a constant temperature chamber at about



25° C. and 60 to 70 per cent relative humidity. They were examined after 24 hours, and subsequently over a period of a week or more.

The criterion of death was the inability of the insect to walk upon a flat surface. Preliminary tests showed that insects which could not walk after treatment nearly always failed to recover. Usually they died in a few days. When disturbed, *Tribolium* often feigns death; and it was therefore necessary to test each individual, which did not walk at once, by applying a slight pressure to the dorsum with the handle of a camel's hair brush. Examinations of each sample were made until two or three successive concordant results were obtained.

The tests of each day were accompanied by one or more control samples of 50 insects removed at random from the culture with the samples which were used in the experiments. Of nearly 7,000 control insects employed in the experiments, only 0.4 per cent died during the period of observation (average 8 days). This very low percentage of mortality is considered negligible insofar as these experiments are concerned.

#### THE CALCULATION OF THE RESULTS

The time required for one of the compounds to kill 50 per cent of the insects at constant concentration and varying exposure time was determined by calculation from the toxicity data. Sufficient tests were made to define the course of toxic action from the region where few insects are killed to the region of about 100 per cent mortality.

A smooth curve fitting these data is typically sigmoid in shape. As the portion of this curve between 25 and 75 per cent mortality is essentially a straight line (21), it is best fitted by the equation for a straight line. The regression formula, employed by statisticians, is such an equation, and its use furnishes a means of closely estimating, from the experimental data, the position of the point of 50 per cent mortality. The regression lines were calculated by the method of Wallace and Snedecor (23). Only those values of time or concentration corresponding to mortalities in the range of 25 and 75 per cent were used in the calculations.

When the time to kill 50 per cent of the insects with the first compound was ascertained, toxicity data for the second compound were obtained by holding this time constant and by varying the concentration. A regression line was then calculated from these toxicity data. The ratio of the calculated 50 per cent points of the two compounds gives the relative toxicity at the particular time level.

The results are summarized in table 2, and are shown graphically in figures 2, 3, 4 and 5.

#### DISCUSSION

The results show that the concentrations of pyridine and of nicotine, expressed as milligrams per liter to kill 50 per cent of the insects, vary inversely with the time. The relation, however, is not linear, the slope of the curve (for pyridine) being steep in the region of high concentration and less steep beyond the 120 minute line (Fig. 6).

In order to compare the toxicities of the two compounds at the same concentration levels, it is convenient to plot the 50 per cent points against time and concentration on a semi-logarithmic grid. The times now lie on a logarithmic scale, the concentrations on an arithmetical scale; and the dis-

TABLE 2. *Toxicity of gaseous pyridine and nicotine to adults of Tribolium confusum at 25° C.*

Compound	Concentration milligrams/ liter	Time minutes	Relative humidity percentage	Mortality percentage
Pyridine	8.2 <sup>1</sup>	360	0	25
	8.8 <sup>1</sup>	360	0	50
	9.4 <sup>1</sup>	360	0	75
	6.7 <sup>2</sup>	208	0	25
	7.6 <sup>2</sup>	208	0	50
	8.5 <sup>2</sup>	208	0	75
	11.2 <sup>1</sup>	133	0	25
	12.9 <sup>1</sup>	133	0	50
	14.6 <sup>1</sup>	133	0	75
	10.3	54.1 <sup>1</sup>	68	25
	10.3	87.6 <sup>1</sup>	68	50
	10.3	121.1 <sup>1</sup>	68	75
	10.3	40.9 <sup>1</sup>	60	25
	10.3	74.1 <sup>1</sup>	60	50
	10.3	107.4 <sup>1</sup>	60	75
	17.0	35.4 <sup>1</sup>	0	25
	17.0	41.8 <sup>1</sup>	0	50
	17.0	48.2 <sup>1</sup>	0	75
	22.8 <sup>1</sup>	20	0	25
	27.7 <sup>1</sup>	20	0	50
	32.5 <sup>1</sup>	20	0	75
	115.9	4.4 <sup>2</sup>	0	25
	115.9	7.1 <sup>2</sup>	0	50
	115.9	9.8 <sup>2</sup>	0	75
Nicotine	.190 <sup>1</sup>	360	0	25
	.228 <sup>1</sup>	360	0	50
	.265 <sup>1</sup>	360	0	75
	.139 <sup>1</sup>	300	0	25
	.159 <sup>1</sup>	300	0	50
	.179 <sup>1</sup>	300	0	75
	.26	86.4 <sup>1</sup>	38	25
	.26	201.6 <sup>1</sup>	38	50
	.26	316.8 <sup>1</sup>	38	75
	.220 <sup>1</sup>	175	0	25
	.275 <sup>1</sup>	175	0	50
	.328 <sup>1</sup>	175	0	75
	.42	100 <sup>1</sup>	0	25
	.42	133 <sup>1</sup>	0	50
	.42	166 <sup>1</sup>	0	75

<sup>1</sup>Values calculated from regression equation.<sup>2</sup>Estimated values.

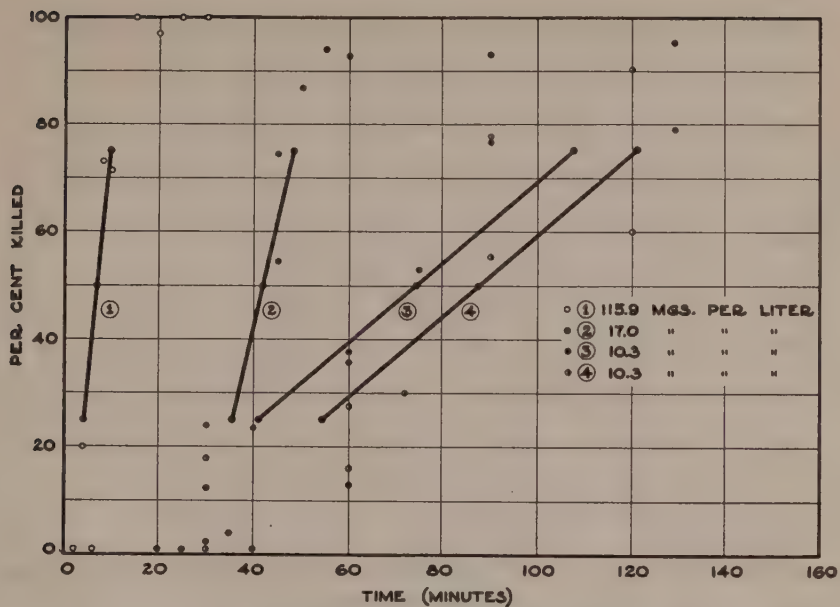


Fig. 2. Toxicity curves for pyridine at constant concentration. The regression lines (in black) cover approximately the linear portions of the curves. Each point represents the mean of two samples of 50 insects.

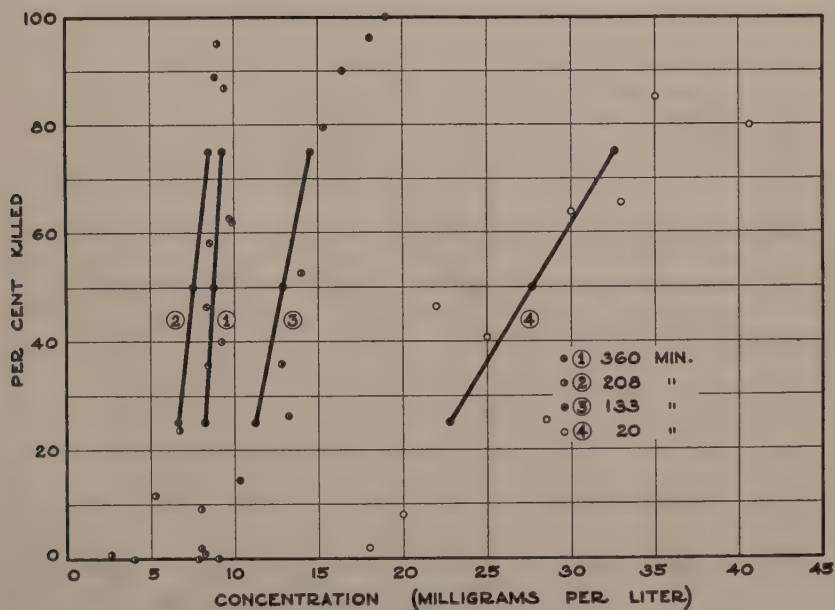


Fig. 3. Toxicity curves for pyridine at constant time.

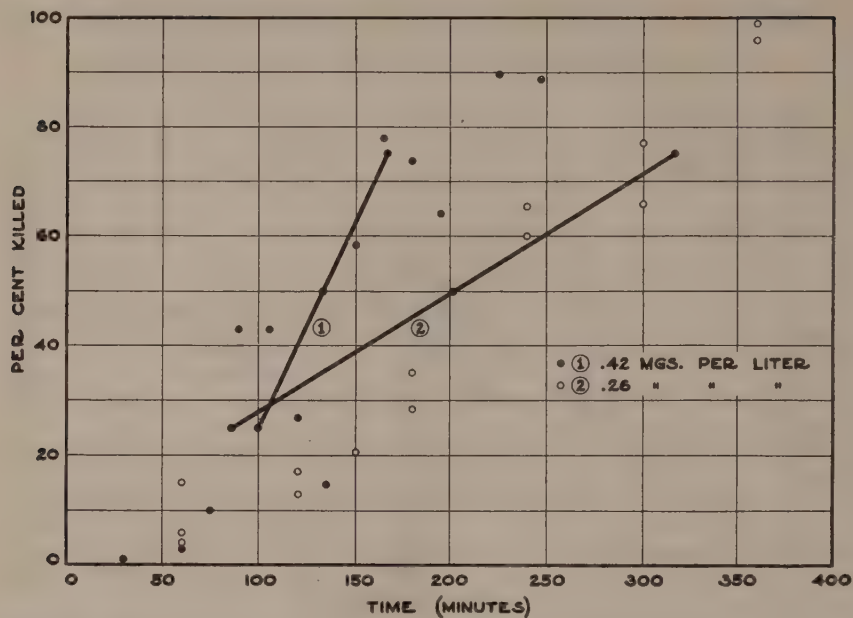


Fig. 4. Toxicity curves for nicotine at constant concentration.

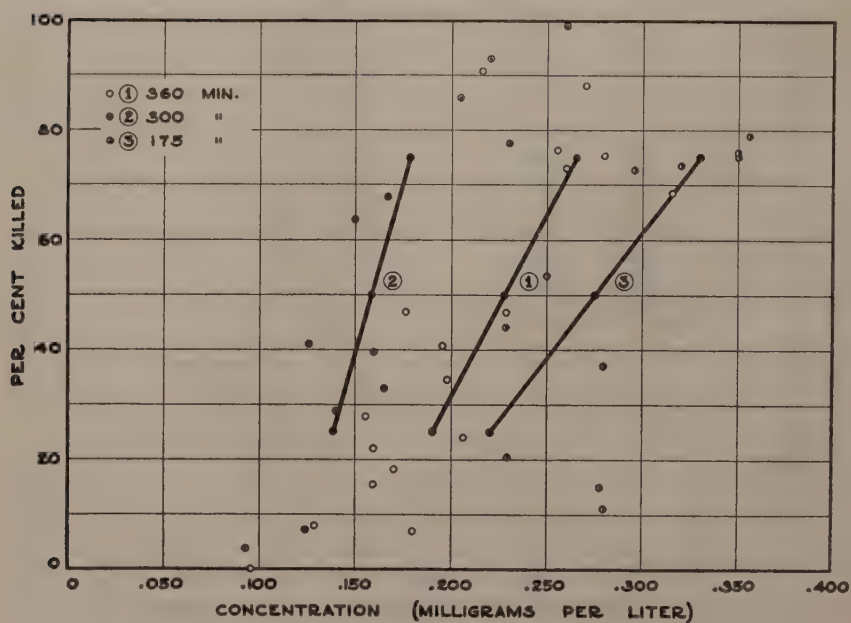


Fig. 5. Toxicity curves for nicotine at constant time.



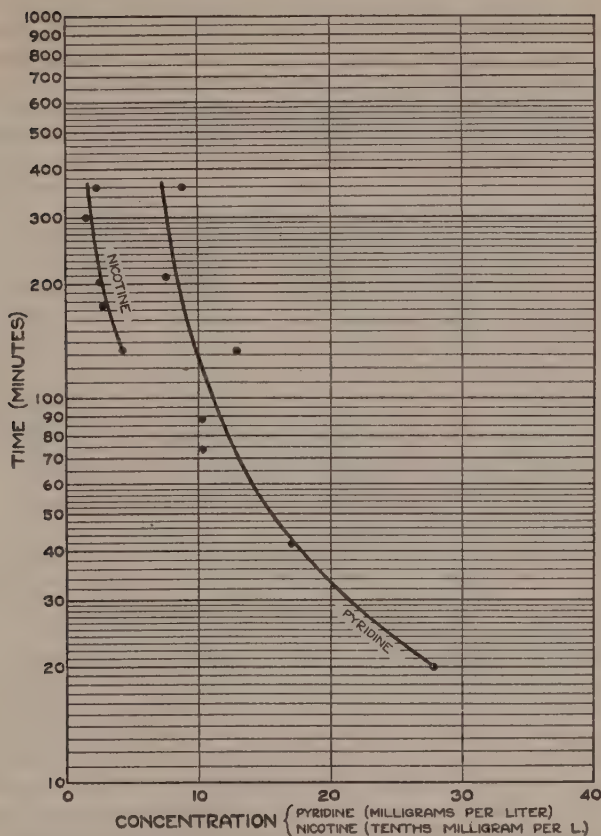


Fig. 6. Semilogarithmic plot of the points of 50 per cent mortality for pyridine and nicotine showing relative toxicity at various time-concentration levels.

tance between the two curves at any comparable time-concentration level gives at sight the relative toxicity. This is shown in figure 6, where at 130 minutes, nicotine is 22 times as toxic as pyridine; at 180 minutes, 29 times; at 240 minutes, 35 times; at 300 minutes, 27 times; at 360 minutes, 44 times. The mean for the five time levels is 31. When a straight line is fitted to the observations in the flat region of the curves the mean of the ratios for the 120, 240 and 360 minute time levels is 28. The relation in this region is apparently not quite linear, but the estimate of 31 taken from figure 6 is probably a fairly close approximation of the ratio of the toxicities of the two compounds.

The validity of the calculations of the 50 per cent points was checked by duplicating the toxicity curves for pyridine at a fixed concentration (10.3 mg./liter). In the first series of experiments, the 50 per cent point was found by calculation to be 87.6 minutes. Four months later, a second series of experiments at the same concentration gave 74.1 minutes, a reduction of about 15 per cent. The agreement, however, is reasonably good

when viewed in relation to the entire curve (Fig. 6). In another series of experiments, with pyridine, made 22 days after the first, a concentration of 7.6 mg./liter for 208 minutes gave mortalities ranging in three tests from 42.9 to 68.8 per cent, mean 55.3 per cent. Three tests made with nicotine, at 0.26 mg./liter, 38 days after the first, but for 208 instead of 201.6 minutes, gave a range of mortalities from 32.6 to 66.7 per cent, mean 53.2 per cent. These experiments demonstrate that the population of *Tribolium* from which the experimental samples were drawn behaved quite uniformly to these toxic gases, and that experimental results with the same population can be approximately duplicated after a considerable lapse of time.

The maintenance of a constant relative humidity added some mechanical difficulties to the experimental procedure, and when rather wide differences in relative humidity showed little, if any, effect on the results, the air used in all experiments was dried to zero per cent. In table 2 and figure 6 it will be seen that the 50 per cent points from experiments in which the relative humidity was zero per cent are no more variable than are those in experiments in which the relative humidity was 38, 60 or 68 per cent. From a study of the toxicity of hydrogen cyanide on several species of insects, Brinley and Baker (1) found that relative humidity had no observable effect on the toxicity of that compound.

The toxicity data for both compounds enabled one to determine whether the effects are reversible, i. e., whether the compounds show any marked anesthetic properties towards this insect. By comparing the percentage of mortality of each test 24 hours after exposure with the percentage of mortality of the final observation (usually made 7 days later), anesthetic effects were readily ascertained. In the experiments with pyridine, the number of cases of recovery after the initial observation about equals the combined number in which there was no change and in which the percentage of mortality had increased. Recovery after exposure to this compound was therefore not a noticeable phenomenon. In nicotine, however, it was more pronounced, 68 per cent of the experiments showing a decrease from the initial mortality in the final observation. The percentage of individuals recovering was also greater in the nicotine experiments, especially in some of those in which the exposure time was rather long. Reversibility to the action of paradichlorobenzene in mites, *Tyroglyphus longior*, has been reported by Jewson and Tattersfield (12) and in *Tribolium confusum* by Lehman (13). Instances of marked reversibility from the effects of carbon disulphide have been reported by Hamlin and Reed (7) in the Indian meal moth, *Plodia interpunctella* (Hübner) and in the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.).

The selection of a criterion of death, which measures changes in the organism so profound that death invariably follows, and which still can be read to a sharp "end point," is often a difficult problem. An apparently satisfactory "end point" sometimes changes when the organism is subjected to widely varying concentrations of a toxic compound, and it is liable to even greater change as one passes from compound to compound. A variation of the latter type has been observed in the present series of experiments. The beetles that survived treatment with nicotine were invariably more sluggish in their movements than were those that had been subjected to pyridine. Though able to walk, they moved more slowly, and usually required a greater tactile stimulus to provoke movement than did

the individuals treated with pyridine. It is therefore probable that nicotine is slightly more toxic than its 50 per cent points indicated.

It is not to be expected that the relative toxicity of pyridine and nicotine, derived from these experiments, will hold for all species of insects. Certain species are known to be much more resistant to toxic compounds than others; and most coleopterous insects will probably be classed among the more resistant groups. It is only through toxicity studies on many insects and many compounds that a complete knowledge of the resistance of insects to toxic substances will be built up.

#### SUMMARY AND CONCLUSIONS

1. The relative toxicity of gaseous pyridine and nicotine to the adult confused flour beetle, *Tribolium confusum* Duval, has been determined.

2. The toxicity of the gases was determined in a closed apparatus, the essential feature of which was the presence of glass throughout the portions in contact with the gas. Gas concentration was regulated by flow meters.

3. The relative toxicity of pyridine and nicotine is approximately the same at all time-concentration levels.

4. At 25° C., nicotine is about 31 times as toxic as pyridine to *T. confusum*.

5. Neither pyridine nor nicotine showed marked anesthetic properties in these experiments. Partial recovery was more pronounced from nicotine than from pyridine.

#### LITERATURE CITED

1. BRINLEY, F. J., AND R. H. BAKER  
1927. Some factors affecting the toxicity of hydrocyanic acid for insects. Biol. Bul. 53(3):201-207.
2. BURN, J. H.  
1930. The errors of biological assay. Physiol. Reviews, 10(1):146-169.
3. CRAIG, L. C.  
1931. Insecticidal action in the nitrogen heterocyclics. Iowa State College Jour. Sci., 5(4):327-330.
4. ———, AND C. H. RICHARDSON  
1930. The calibration of flow meters for the measurement of insecticide gases. Jour. Econ. Ent., 23(6):988-991.
5. DE ONG, E. R.  
1918. The relation between the volatility and toxicity of nicotine in sprays and dusts. Jour. Econ. Ent., 16(6):486-493.
6. FISHER, R. A.  
1924. In Tattersfield, F., and H. M. Morris. An apparatus for testing the toxic values of contact insecticides under controlled conditions. Bul. Ent. Research 14(3):223-236.
7. HAMLIN, J. C., AND W. D. REED  
1927. Insect revival after fumigation. Jour. Econ. Ent., 20(2):400-427.
8. HARLAN, W. R., AND R. M. HIXON  
1928. Volatility of nicotine. Ind. Eng. Chem., 20(7):723-725.

9. ———— AND ————  
1928. Chemical testing of nicotine dusts. Iowa State College Jour. Sci., 2(4):313-316.
10. ———— AND ————  
1930. Catalytic reduction of nicotine and metanicoline. Jour. Amer. Chem. Soc., 52(8):3385-3388.
11. HOLT, J. J. H.  
1916. The cockroach: Its destruction and dispersal. Lancet, 190(1):1136-1137.
12. JEWSON, S. T., AND F. TATTERSFIELD  
1922. The infestation of fungus cultures by mites. (Its nature and control, together with some remarks on the toxic properties of pyridine.) Ann. Appl. Biol. 9(3 and 4):213-240.
13. LEHMAN, R. S.  
1930. A comparison of the toxicity of paradichlorobenzene and naphthalene to the confused flour beetle (*Tribolium confusum* Duv.) (Coleoptera). Jour. Econ. Ent., 23(6):958-966.
14. MCCLINTOCK, C. H., H. C. HAMILTON AND F. B. LOWE  
1911. A further contribution to our knowledge of insecticides: Fumigants. Jour. Am. Public Health Assn., 1(4):227-260.
15. MOORE, W.  
1917. Volatility of organic compounds as an index of the toxicity of their vapors to insects. Jour. Agric. Research, 10(7):365-371.
16. ———— AND S. A. GRAHAM  
1918. Toxicity of volatile organic compounds to insect eggs. Jour. Agric. Research, 12(9):579-587.
17. NEIFERT, I. E., AND G. L. GARRISON  
1920. Experiments on the toxic action of certain gases on insects, seeds and plants. U. S. Dept. Agr., Bul. 893, 16 p.
18. ———— AND F. C. COOK, R. C. ROARK, W. H. TONKIN, E. A. BACK AND R. T. COTTON  
1925. Fumigation against grain weevils with various volatile organic compounds. U. S. Dept. Agr. Bul. 1313, 40 p.
19. STRAND, A. L.  
1930. Measuring the toxicity of insect fumigants. Ind. Eng. Chem., Analytical Ed., 2(1):4-14.
20. TATTERSFIELD, F., AND A. W. R. ROBERTS  
1920. Influence of chemical constitution on the toxicity of organic compounds to wireworms. Jour. Agric. Sc., 10(2):199-232.
21. TREVAN, J. W.  
1927. The error of determination of toxicity. Proc. Royal Soc. London, 101(B):483-514.
22. TRILLAT AND LEGENDRE  
1909. Etude sur la toxicite des vapeurs de quelque substances chimiques sur les moustiques. Hygiene générale et appliquée 4(9):542-546. (Original not seen.)
23. WALLACE, H. A., AND G. W. SNEDECOR  
1931. Correlation and machine calculation. Official Publication, Iowa State Coll., 30(4), 71 p. (Revised).



# QUANTITATIVE, BIOMETRIC AND HOST-PARASITE STUDIES ON *EIMERIA MIYAIRII* AND *EIMERIA SEPARATA* IN RATS<sup>1</sup>

E. R. BECKER, PHOEBE R. HALL AND ANNA HAGER

*From the Department of Zoology and Entomology, Iowa State College*

Accepted for publication March 31, 1932

The rat lends itself exceedingly well for use as a host in which to cultivate coccidia for the study of a number of general problems relative to these microorganisms. In the first place, it is an animal which is easily handled in the laboratory. Second, it is comparatively prolific and usually available. Third, much information has been accumulated concerning the normal growth rate of this animal and the effect of diet on its growth. Fourth, the animal's habit of dropping its egesta in the form of pellets is a distinct advantage in collecting the oocysts. Fifth, under the proper conditions it develops a prompt and complete, though not lasting, immunity to the coccidian parasites. And sixth, when the animals are properly handled, the chances of accidentally spreading the infection where it is not wanted—as in the breeding cages or among the controls—are almost nil.

The rat is the host for at least two well-defined species of coccidia—*Eimeria miyairii* and *E. separata*. The name of the former was proposed by Ohira (1913), but his paper is unavailable to us and it is impossible to tell very much about the characters of the coccidium from the abstract. Following Pérard (1926), however, we are tentatively accepting Ohira's designation for the coccidium of the rat with the larger oocysts. *E. separata*, the species with the smaller oocysts, was described in brief by Becker and Hall in 1931. Frequent comparisons of the two species will be made throughout the course of this paper. A summary of the characters of the two species appears in table 1.

Strains of the two parasites were originally obtained from a mixed infection in a wild Norway rat captured in the vicinity of our laboratory. The oocysts from the cecum of this animal were cultured in four per cent potassium dichromate solution for five days. Then some of the culture material was fed to laboratory rats. It was noted that at the end of five or six days small oocysts were present in the feces, and that on the seventh or eighth days larger oocysts made their appearance. By collecting and culturing the oocysts which appeared on the fifth day and feeding them to clean<sup>2</sup> rats, a pure strain of the smaller forms was obtained. A pure strain of the larger forms was obtained by collecting the oocysts which appeared on the eleventh day after the infective feeding. The first attempt was successful with the smaller species, but it was necessary to pass the organisms through several rats in order to obtain a pure strain of the larger.

All but a few of the rats used in the experiments were raised in our

---

<sup>1</sup>This work was supported by a grant from the Rockefeller Fluid Research Fund at Iowa State College.

<sup>2</sup>Used here to mean uninfected or never previously infected.

laboratory. The stock of rats employed was of exceedingly mixed heredity, for it came originally from at least four different sources. Whether this accounts for the extreme variability in degree of susceptibility to the parasites which we met with remains to be seen.

TABLE I. *A comparison of the characteristics of E. miyairii and E. separata*

	<i>E. miyairii</i>	<i>E. separata</i>
Shape of oocysts	Ovoidal	Ellipsoidal, seldom ovoidal
Range in size of oocyst	16.2 to 26.4 $\mu$ x 13.4 to 21.3 $\mu$	12.8 to 19.4 $\mu$ x 11.2 to 17.2 $\mu$
Mean size of oocyst	22.5 $\mu$ x 17.8 $\mu$	16.06 $\mu$ x 13.85 $\mu$
Average $\frac{L}{W}$ of oocysts	1.265	1.16
Oocystic residual body	absent	absent
Sporocystic residual body	present	present
Sporulation time	65 to 72 hrs.	27 to 36 hrs.
Prepatent period	7 to 8 days	5 to 6 days
Patent period (single inoculation)	5 to 6 days	3 to 4 days
Patent period (repeated inoculation)	5 to 8 days	4 to 6 days
Av. no. oocysts discharged in patent period (repeated inoculations)	5,421.3 x 10 <sup>4</sup>	245.9 x 10 <sup>4</sup>

#### METHODS

There is one point which we wish to emphasize especially: None of the rats used in the experiments here reported had a previous infection with coccidia, accidental or otherwise. The young rats were examined for oocysts by means of the salt flotation technique every third or fourth day from the time they were about three weeks old. Furthermore, either they were kept in cages with clean shavings on the bottom, the cages being changed and thoroughly dried every third day, or they were kept on screens. All food and water containers were sterilized with hot water daily. There were only two accidental outbreaks. One young rat in a breeding cage with nine others became infected when somebody carelessly admitted a large number of flies into the laboratory, and one control rat became infected. The latter infection is to be explained by the fact that the leavings of the infected rats were fed to some controls—a practice which was immediately discontinued. Had any of the growing rats become infected, we would have detected it during the routine examinations.

The young rats, after weaning, were raised on a modification of the Steenbock growth ration, and milk and water *ad libitum*. The ration used was as follows:

Yellow corn meal .....	76.0 lbs.
Linseed oil meal .....	16.0 "
Commercial casein .....	5.0 "
Ground alfalfa .....	2.0 "
NaCl .....	0.5 "
CaCO <sub>3</sub> .....	0.5 "
Dried buttermilk .....	12.0 "

The animals were given only the grain mixture and water while on experiment.

The rats on experiment were kept individually in specially made cages of hardware cloth fitted over 9 in. x 12 in. aluminum or enamel pans, so that the fecal pellets dropped through the meshes of the floor into water in the pan below. The food and water containers were so designed that very little of their contents dropped from them.

The rats were infected by feeding them either a few ccm. of milk or a small cube of bread containing diluted culture material of a known content of sporulated oocysts. We found this method far more satisfactory than etherizing the rats and force-feeding them through a catheter.

Our technique for counting the oocysts was simple, and we believe, more accurate than some of the more complicated ones. The water in the pans containing the pellets was poured into a beaker and permitted to soak for several hours. Then the material was thoroughly homogenized with an electric mixer. This process usually disintegrated all but the solid undigested particles of food, although a little assistance with a flat-headed stirring rod was occasionally required. The material was poured into a 200 cc. volumetric flask and made up to the mark with water. Then it was again thoroughly agitated with the mixer. The coarser particles were removed by dashing the material through screens in such a fashion that there were no accumulated particles to screen out oocysts. A small amount of the suspension was at once transferred to a haemocytometer of 0.1 mm. depth, and the oocysts in a volume of 0.9 cmm. were counted. The counts were made twice if they agreed closely; if not, four times. After the number of oocysts in 1.8 cmm. or 3.6 cmm. was determined, the daily yield was easily calculated.

Measurements of the oocysts were made by means of the microscope and the camera lucida. We found a 20x eyepiece and the 44x high dry lens the most satisfactory combination. It was noted that oocysts often stand on end and appear to be more or less round. By gently tapping on the cover glass such specimens can be made to shift their position so as to reveal their true length. As a matter of fact, we did not find a single round oocyst of *E. miyairii*, although a very few of *E. separata* were almost round. The specimens were floated in sugar, transferred to a glass slide by means of a flat-headed solid glass rod, and measured in the order in which they were encountered so that there was no selection of sizes. Generally at least fifty were measured from each day's collection, although in a very few cases it was impossible to find that many on the last day of the patent period.

## THE SIZE OF THE OOCYSTS

It was, of course, highly desirable to ascertain the exact size of the oocysts of the two species through the measurement of a large number of specimens of each. There was, however, a much broader problem involved. Certain recent authors—notably Boughton (1930), Fish (1931) and Tyzzer (1932)—deprecate oocyst measurements in the describing of new species. Boughton found that the oocysts of *Isospora lacazei* from infected sparrows varied considerably in size from time to time. He admits, however, that he may have been dealing with more than one species of parasite. Fish (1931) found that the oocysts of *Eimeria tenella* from chickens tended to become longer and broader as the infection progressed, so that a peak was usually reached before the infection elapsed; but the oocysts on the final day were larger than those on the first day. He states: "Size is, at best, an unreliable specific criterion. . . . Specific biometric data, obtained at any one time during an infection may be entirely misleading when applied to oocysts from another host harboring the same species of parasites, or to oocysts from the same host at another time during the same infection." There is probably no doubt that in the past too much emphasis has been placed upon mere size as a specific character, and that there is a certain amount of justification in this attitude. Since the above-named workers all employed avian hosts, it seemed that a careful study of the value of measurements on mammalian coccidia would be in order. Moreover, the patent period for both *E. miyairii* and *E. separata* of the rat is comparatively short, and any generalized tendency for oocysts to change their shape, if such exists, would be telescoped into a shorter interval and thereby be more readily detectable.

TABLE 2. Daily mean measurements (in microns) on oocysts from rats harboring both *E. miyairii* and *E. separata*

Day after infective feeding	Rat 10		Rat 11		Rat 12		Rat 13	
	L.	W.	L.	W.	L.	W.	L.	W.
5	15.4	12.9	16.2	13.4	16.2	14.0	15.5	12.9
6	17.1	14.1	16.4	13.9	17.7	14.6	16.9	14.0
7	16.2	12.7	16.4	13.6	18.3	15.2	17.2	14.5
8	24.6	18.8	22.0	17.7	22.0	17.5	21.6	17.4
9	21.1	16.8	22.0	17.7	21.3	17.2	22.2	17.6
10	21.4	17.5	22.2	17.9	21.3	17.1	21.5	17.1
11	21.9	17.9	21.9	18.0	21.7	17.5	21.5	17.9
12	22.5	18.3	21.1	16.8	21.8	18.2	21.0	16.9

Four sets of measurements were made with the same number of objectives in mind. The first oocysts measured were from four rats which had been infected once with a small number (100 to 200) of the sporulated oocysts taken originally from the cecum of a wild rat naturally infected with the two species. The second set of measurements was made to determine how well one might select oocysts of only one species (*E. miyairii*) from material known to contain oocysts of two species. For this experiment four rats were infected by feeding them daily for ten days about



1,500 sporulated oocysts from the original mixed culture. The third set was made on oocysts from four rats infected by feeding each 425 sporulated oocysts from a pure culture of *Eimeria miyairii*. The last set of measurements was of oocysts discharged from three rats infected exclusively with *Eimeria separata*. These rats were infected by feeding each 1,500 sporulated oocysts daily for five days. The results are summarized in tables 1, 2, 3 and 4. With very few exceptions, as noted above, each daily mean measurement is based on 50 specimens, except in the case of *E. separata*, for which the daily number was 25.

Measurements of oocysts such as those presented in table 2 led us to suspect that our original material from the wild rat consisted of two species. The means for the first three days of the patent period varied from 15.4  $\mu$  to 18.3  $\mu$  for length and from 12.7  $\mu$  to 14.6  $\mu$  for width. The means for the last five days of the patent period varied from 21.0  $\mu$  to 24.6  $\mu$  for length and 16.8  $\mu$  to 18.8  $\mu$  for width. If the measurements of the entire 1,600 oocysts were plotted, they would make a bimodal curve. The smaller forms only were present on the fifth and sixth days, and on the seventh day they constituted all but an almost negligible fraction of those measured. On the last five days of the patent period the oocysts were almost exclusively those of *E. miyairii*. The reason for the presence of only *E. separata* early in the infection was due to its short prepatent period. Its almost total lack of effect upon the measurements after the seventh day is due to the facts that it does not appear in the enormous numbers encountered in the other species and that the patent period is characteristically not over four days after a single infective dose of the sporulated oocysts. Certainly in this instance we have demonstrated beyond a doubt that there are instances where oocyst measurements have a distinct value.

TABLE 3. Daily mean measurements in microns of oocysts selected for *E. miyairii* from rats infected with both *E. miyairii* and *E. separata*

Day after infective feeding	Rat 22		Rat 26		Rat 27		Rat 31	
	L.	W.	L.	W.	L.	W.	L.	W.
7	21.0	16.5	20.6	15.0	20.1	16.3	18.4	15.7
8	21.0	16.5	20.0	16.2	20.6	16.2	19.9	16.4
9	20.4	16.1	20.6	16.2	21.1	16.4	20.5	16.3
10	21.5	16.8	21.0	16.5	21.9	17.0	22.7	17.9
11	21.0	16.8	21.1	16.3	21.5	17.3	21.4	16.8
12	21.0	17.1	20.4	16.5	23.1	17.1	21.7	17.2
13	23.3	18.7	Neg.	Neg.	Neg.	Neg.	21.9	18.0
14	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Mean of means	21.3	16.9	20.6	16.2	21.4	16.7	20.9	16.9

In the second series of measurements, presented in part in table 3, the population of oocysts was mixed throughout the infection. The effort to select for measurement only the oocysts of *E. miyairii* was fairly successful, considering that the only criterion was the judgment of the manipulator as to the size and shape of the oocysts. The average size of all the oocysts

TABLE 4. Daily mean sizes and standard deviations in microns of oocysts from pure *E. miyairii* infections in rats (*L*, length; *W*, width; *S. D.*, standard deviation)

Day	Rat 145				Rat 146				Rat 147				Rat 148			
	L.	S.D.	W.	S.D.	L.	S.D.	W.	S.D.	L.	S.D.	W.	S.D.	L.	S.D.	W.	S.D.
8	23.2	1.55	18.6	1.26	21.4	2.00	17.7	1.54	21.0	2.80	17.1	1.34	21.2	2.42	17.0	1.10
9	23.6	2.39	18.6	1.13	22.1	3.59	17.6	1.05	22.3	1.16	18.0	1.01	23.3	1.33	18.1	.93
10	23.8	1.25	18.3	.98	23.1	1.32	18.2	1.35	22.2	1.07	17.8	.81	22.3	1.08	17.8	1.20
11	22.3	1.86	17.6	.92	21.9	1.14	17.6	1.03	22.0	1.50	17.5	1.26	22.5	1.02	17.8	1.01
12	23.5	1.90	18.4	1.87	23.0	1.46	17.9	1.04	22.4	.87	17.8	1.09	22.6	1.17	18.0	1.05
13	23.0	1.40	17.8	1.31	22.5	1.53	17.5	.99	22.8	1.44	17.7	1.03	22.4	1.3	17.5	.92
14	23.3	1.96	18.4	1.73	23.0	1.40	18.0	1.12								
15	23.0	1.33	18.2	.91	22.5	1.20	18.1	1.04								
16	22.6	.80	18.0	.96	22.9	1.90	17.5	1.10								
17	23.6	—	17.8	—												
Mean of means	23.2		18.2		22.3		17.8		22.1		17.7		22.4		17.7	

measured was  $21.5 \mu$  by  $16.7 \mu$ , whereas the mean size of *E. miyairii* in pure infections is  $22.52 \mu$  by  $17.804 \mu$ . Evidently a few of the larger specimens of *E. separata* were included in the measurements, but the results are highly gratifying in showing that under certain conditions one species in a mixed infection may be identified on the basis of oocyst size and, to some extent, shape.

The third series of measurements, presented in part in table 4, involved altogether 1,485 oocysts of *E. miyairii*. From this study it was determined that these bodies range in length from  $16.24 \mu$  to  $26.43 \mu$  and in width from  $13.37 \mu$  to  $21.34 \mu$ . The mean size, as computed from the classes and frequencies shown in figure 1, is  $22.525 \pm .027 \mu$  by  $17.804 \pm .020 \mu$ , and the ratio of the two dimensions is 1.265. The standard deviation for length is  $1.50 \mu$ ; for width,  $1.17 \mu$ . The table shows no constant trend of the oocysts toward a larger or smaller size as the infection progresses. For any one rat the mean values for length or width during any one day fall within the range of the standard deviation for any other day. Also, it is to be noted that the daily means for length vary only from  $21.0 \mu$  to  $23.8 \mu$ ; for width, from  $17.0 \mu$  to  $18.6 \mu$ .

The curves constructed to represent the total number of oocysts (Fig. 1) show a skewness to the right for both length and width. The mode of the former is at  $22.5 \mu$ , the latter at  $17.5 \mu$ . The curves for the individual rats (not shown here) show modes for length and width as follows: Rat 145, 23.5 and 18.5; Rat 146, 22.5 and 17.5; Rat 147, 22.5 and 17.5; Rat 148, 21.5 and 17.5. It is apparent that the size and shape index of the oocysts vary but slightly in the different hosts.

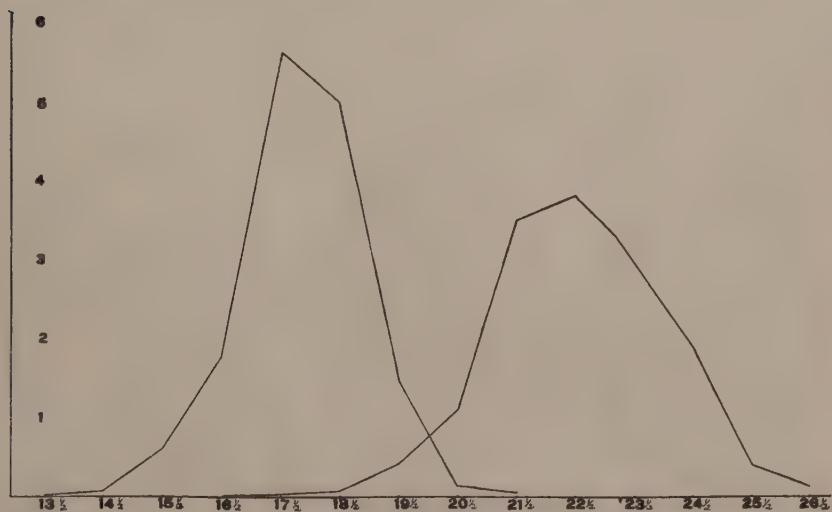


Fig. 1. Frequency curves for length and width of *E. miyairii* (Ordinate, number of oocysts in hundreds; abscissa, class groupings in microns).

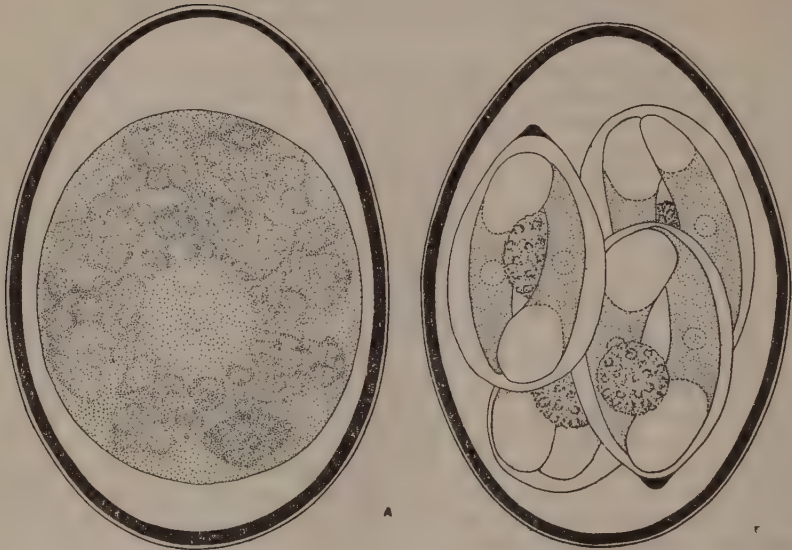


Fig. 2. a, unsporulated oocyst, *E. miyairii*; b, completely sporulated oocyst of same.



Fig. 3. a, unsporulated oocyst of *E. separata*; b, oocyst of *E. separata* containing immature spores; c, completely sporulated oocyst of *E. separata*, showing characteristic arrangement of sporocysts.



Fish (1931) observed that the shape index of *Eimeria tenella* was not constant throughout the entire size range of oocysts, for the longest were not in all cases the widest. In order to determine whether this was a rule which would apply also to *E. miyairii*, the average ratio of length to width was ascertained for all oocysts measured from Rat No. 145 which lay between 18 and 20  $\mu$ , inclusive, and between 25 and 26  $\mu$ , inclusive. The ratio for the shortest 27 oocysts was 1.182; for the longest 27 oocysts, 1.419. For Rat No. 146, the ratio for the 52 oocysts between 16 and 20  $\mu$  in length was 1.183; for the 58 oocysts between 24 and 26  $\mu$  in length, 1.363. The shape index for the 1,485 oocysts measured from four rats, as was previously mentioned, was found to be 1.265. Thus, the shortest oocysts of the population tend to have a smaller shape index than the average; and the longest tend to have a larger shape index than the average.

Use was made of the formula  $r = \frac{\sum D_x D_y f}{N\sigma_x \sigma_y}$  in order to determine

whether there was a definite positive correlation between length and width. All the oocysts measured from Rats No. 145 and 146 were grouped into classes at intervals of one micron in the construction of a correlation table. The  $r$  values for Rats 145 and 146 were .29 and .31, respectively. These values indicate a definite correlation between length and width, but admittedly are not so high as was expected.

The measurements on *E. separata*, shown in part in table 5, yielded the following values: Largest oocyst, 17.2  $\mu$  by 19.4  $\mu$ ; smallest oocyst, 11.2  $\mu$  by 12.8  $\mu$ ; mean size of all oocysts (400 were measured), 16.06  $\mu$  by 13.85  $\mu$ . The ratio of length to width was 1.16. It is evident here also that the oocysts exhibit no tendency toward a larger or smaller size as the infection progresses. The measurements here submitted vary somewhat from those appearing in the original description of the species. The only conceivable explanation is that by passing the organisms through a further

TABLE 5. Daily mean sizes, in microns, of oocysts from pure *E. separata* infections in rats

Day	Rat F		Rat G		Rat H	
	L.	W.	L.	W.	L.	W.
6	16.3	14.1	17.3	15.1	15.9	13.8
7	15.7	13.6	16.6	14.5	16.0	13.8
8	15.5	13.4	15.3	13.6	17.2	14.6
9	15.1	13.0	15.8	13.6	16.8	14.0
10	15.3	13.1	16.0	13.9	Neg.	Neg.
11	15.1	13.1	16.3	14.0	Neg.	Neg.
Mean of means	15.5	13.38	16.22	14.12	16.48	14.05
$\frac{L}{W}$	1.16		1.15		1.17	

series of rats, and continually selecting the first to appear as infective material for the next passage, an early appearing strain with smaller and shorter oocysts was unintentionally isolated.

#### THE NUMBER OF OOCYSTS PRODUCED DURING INFECTIONS WITH

E. MIYAIRII

There were three principal reasons for determining the number of oocysts produced during an infection. First, it was necessary to establish the normal number as a basis for future experimental attempts to influence the infection through artificial means. Second, a comparison of the oocysts yielded in pure infections of the two species at our command was highly desirable. And third, the variations in the yields of oocysts from different rats observed early in the course of our work prompted us to investigate the extent of this natural host variability. All the rats on this experiment were fed 1,500 sporulated oocysts (out of the same bottle) daily for five days. The first oocysts appeared on the seventh or eighth days, and the last on the thirteen, fourteenth or fifteenth days. All of the rats became entirely immune at the end of the infection, and efforts to reinfect them immediately were unsuccessful. Hence, the figures in table 6 really represent the numbers of oocysts produced in infected rats during the period of acquiring total immunity.

A statistical analysis of the oocyst yields presented in table 4 shows that the average yield per rat was  $5,421.3 \pm 321 \times 10^4$  oocysts. The yields ranged from  $1,410 \times 10^4$  to  $16,922 \times 10^4$  oocysts. The standard deviation for all the rats was found to be very high— $3296.08 \times 10^4$ ; the coefficient of variation was, roundly, 60 per cent. The mean yield for the 30 males was  $6,434.4 \pm 439.4 \times 10^4$ ; for the females,  $3,732.83 \pm 284 \times 10^4$ . A comparison of the yields for the two sexes gives a Diff. means / P.E. diff. value of 5.143.

Unfortunately, the males and females were from different litters, with the exception of rats 135 to 144, which constituted a single litter. Within this one litter the average yields for the two sexes were not so strikingly different. Therefore, it is somewhat doubtful if the suggested difference in the susceptibility of the two sexes represents an actuality.

An inspection of our table showing the daily yields, which is too voluminous to be published here, brings out some points of further interest. Twelve of the 48 rats commenced to eliminate oocysts on the seventh day after the first infective feeding; the remainder, on the eighth day. Four ceased eliminating on the twelfth day, but all of these had begun on the seventh day. Twenty-nine ceased on the thirteenth day; thirteen, on the fourteenth day; and two on the fifteenth day. In two cases the highest yield came on the eighth day; in 32, on the ninth day; in 14, on the tenth day. The daily mean yields and standard deviations, respectively, for all the rats were as follows (expressed in terms of  $\times 10^4$ ): Seventh day, 1.73 and 3.91; eighth day, 92.56 and 85.41; ninth day, 206.23 and 143.06; tenth day, 146 and 105.6; eleventh day, 64.98 and 49.14; twelfth day, 21.69 and 13.65; thirteenth day, 4.71 and 3.55; fourteenth day, 0.644 and 1.444; fifteenth day (mean only), 0.06.

Figured on the basis of percentages, these data mean that on the seventh day of the infection, 0.32 per cent of the total number of oocysts were discharged; on the eighth day, 17.18 per cent; ninth day, 38.28 per cent; tenth day, 27.11 per cent; eleventh day, 12.06 per cent; twelfth day, 4.03 per cent; thirteenth day, 0.78 per cent; fourteenth day, 0.12 per cent; fifteenth day, .011 per cent. About 95 per cent of the total number of oocysts leave the host from the eighth to the eleventh days, inclusive.

Since we weighed all the rats on the day of the first infective feeding, it was possible to make a statistical analysis of the relationship between the weight of the rat and intensity of the infection as measured by the total output of oocysts. Using the correlation formula, an  $r$  value of  $-0.186$  was obtained. This means that there was practically no inverse correlation between the weight of the rats (and hence the age) and the oocyst output. Therefore, the variability in the intensity of susceptibility of the hosts was due to some factor other than the weight (or age) of the rats.

#### THE NUMBER OF OOCYSTS PRODUCED DURING INFECTIONS WITH *E. SEPARATA*

The methods in this study were the same as in that for *E. miyairii*. A much lower oocyst yield occurs in this species. The yields of oocysts from seven rats, which averaged around 80 grams each at the time of the first infection were as follows: Rat A, 2,467,603; Rat B, 1,791,922; Rat C, 1,393,246; Rat D, 2,313,578; Rat E, 3,756,351; Rat F, 3,327,279; Rat G, 3,264,354; Rat H, 1,358,800. The average yield of  $245.9 \times 10^4$  oocysts per rat is but 4.6 per cent of that in the case of *E. miyairii* for which an average yield of  $5,421.3 \times 10^4$  oocysts per rat was demonstrated.

A further examination of our records for the seven rats infected with *E. separata* shows the patent period was six days for six of the rats, five days for one, and four days for one. In all cases discharging of oocysts commenced on the sixth day after the first infective feeding. The average daily production in percentages of the total yields are as follows: Sixth day, 13.6 per cent; seventh day, 32.3 per cent; eighth day, 24.0 per cent; ninth day, 17.1 per cent; tenth day, 8.9 per cent; eleventh day, 4.2 per cent.

#### THE EFFECT OF THE SIZE AND FREQUENCY OF THE INFECTIVE DOSE ON THE NUMBERS OF OOCYSTS DISCHARGED

On September 14, rats 10 to 19, weighing from 70 to 90 grams apiece, were each fed between 100 and 200 sporulated oocysts from a mixed *E. miyairii* and *E. separata* culture. The smaller forms appeared in the feces on September 19 and 20, and the larger ones on the following five days. The total number of oocysts discharged by the individual rats ranged from  $555 \times 10^4$  to  $3,488 \times 10^4$ . The average number per rat was  $1,816.6 \times 10^4$ . Since the numbers of *E. separata* were almost negligible, very little error is involved in considering these figures as representing almost entirely *E. miyairii*. The yields are to be compared with a range of from  $1,410 \times 10^4$  to  $16,922 \times 10^4$  and an average per rat of  $5,421 \times 10^4$  in the 48 rats which

TABLE 6. Sex, weight at time of infection, and total numbers of oocysts produced in white rats infected on five successive days with *E. murrayi*

Rat No.	Sex	Weight	No. oocysts 10 <sup>4</sup>	Rat No.	Sex	Weight	No. oocysts 10 <sup>4</sup>	Rat No.	Sex	Weight	No. oocysts 10 <sup>4</sup>
22	♂	145	1,975	74	♀	113	6,397	127	♂	112	2,722
26	♂	148	5,986	92	♂	92	11,644	128	♂	132	1,994
27	♂	144	8,624	93	♂	82	6,778	129	♂	133	6,489
31	♂	149	4,951	94	♂	72	6,992	130	♂	127	4,510
50	♂	124	3,531	95	♂	86	12,866	131	♂	127	3,306
51	♂	127	4,172	96	♂	88	7,914	132	♂	116	10,311
52	♂	127	3,900	97	♂	81	4,656	135	♀	75	4,622
53	♂	133	9,011	111	♀	99	4,356	136	♀	73	3,533
54	♂	130	2,511	112	♀	110	1,522	137	♂	72	4,200
55	♂	115	2,697	113	♀	101	3,225	138	♂	77	6,100
68	♀	102	1,768	114	♀	115	3,010	139	♂	76	4,144
69	♀	106	2,867	115	♀	113	1,410	140	♂	72	5,667
70	♀	123	1,458	123	♂	127	8,656	141	♂	71	10,300
71	♀	116	2,972	124	♂	102	5,911	142	♀	72	5,911
72	♀	112	3,492	125	♂	114	16,922	143	♀	65	2,156
73	♀	120	4,202	126	♂	102	10,778	144	♀	70	7,144



were inoculated with 1,500 sporulated oocysts of *E. miyairii* daily for five days.

Furthermore, the patent period for *E. miyairii* in the rats infected once was five days; in the case of the rats infected on each of five successive days, five to eight days. The ten rats which were inoculated with the single small dose of oocysts were capable of reinfection almost immediately, save Rat No. 15. This animal yielded only  $555 \times 10^4$  oocysts, and it could not be reinfected. (It was the only rat in our laboratory which had an infection with the tapeworm *Hymenolepis diminuta*). The 48 rats inoculated on five successive days could not be immediately reinfected.

This experiment shows, then, that the size and frequency of the infective dose has an important influence upon the numbers of oocysts produced, the length of the patent period, and immunity. This preliminary work suggests that further and more exacting studies along this line would be desirable.

#### EFFECT OF COCCIDIA ON THE HEALTH OF THE RAT

Pérard claims that *Eimeria miyairii* may produce a severe diarrhea in young culture rats, leading in some cases to death within eight or ten days. None of our 48 experimental rats infected with 1,500 oocysts daily for five days showed any symptoms of disease. We kept records of the weights of the first 23 rats, 16 males and 7 females, throughout the course of the infection. In addition, we maintained 23 control rats, also 16 males and 7 females of as nearly as possible the same weight, rat for rat, as the experimental animals. At the time the experimental rats were given the first sporulated oocysts they showed an average weight of 114.6 gm.; the control rats, 110.1 gm. On the twelfth day of the infection the infected rats showed an average weight of 146.7 gm.; on the same day the control rats showed an average weight of 147.6 grams. Thus the control rats made a gain of 37.5 grams per rat in 12 days. The infected rats gained 32.1 grams per rat in the same period. While the control rats gained 5.4 grams per rat more than the infected ones, this difference proves not to be statistically significant.

There is no doubt, however, that *E. miyairii* is pathogenic in massive doses to rats of all ages. Six rats of about a hundred grams were each fed 55,000 sporulated oocysts, and killed five days later. The lower third of the small intestine of three of them was markedly hemorrhagic, for the lumen was filled with a mixture of blood and partially digested food. The other three showed practically no hemorrhage, but the blood vessels in the wall of the small intestine, cecum, and large intestine were hyperemic. A large male rat of about fifteen months of age died on the fifth day after ingesting 100,000 oocysts of *E. miyairii*. The fate of some parabiotic twins will be mentioned subsequently.

The conclusion, then, is that *E. miyairii* is not highly pathogenic in small doses, but in overwhelming doses (100,000 oocysts) it is fatal to some, though not all, rats.

Our experience with *E. separata* is quite limited, but we have never been able to observe that it had any effect upon the health of a rat. Three 100 gram rats which were fed 50,000 sporulated oocysts each showed no illness or loss of appetite. Further study, however, will be required before it can be stated definitely that it is altogether a non-pathogen.

## HOST-SPECIFICITY STUDIES

Dieben (1924) reported his unsuccessful attempts to infect mice, guinea pigs and rabbits with *Eimeria nieschulzi* (= *miyairii*). Nor could he infect rats with coccidia from rabbits. He could, however, infect *Mus norvegicus* with the parasite from *Mus rattus*, and vice versa, and tame white and spotted rats with parasites from both. Pérard (1927) was unable to make the transfer of *E. schubergi* from mice to rats, or of three species of the rabbit to rats. Kartchner and Becker (1929) could not infect rats and mice with *Eimeria citelli* from the ground squirrel.

We attempted without success to transfer both *Eimeria miyairii* and *E. separata* to 13 coccidia-free white mice and 4 coccidia-free striped ground squirrels (*Citellus tridecemlineatus*). The dose for each animal was 1,500 sporulated oocysts of each species daily for five days. The oocysts were being used successfully at the same time to infect rats, a test which proves their viability. The fecal pellets of the mice and squirrels were thoroughly examined for from five to fourteen days after the inoculation date, but were always negative for oocysts. If such close relatives of the rat can not be infected with the coccidia of the rat, it is extremely unlikely that these microorganisms can grow in any animals except their indigenous hosts.

## IMMUNITY

It has previously been shown that rats vary exceedingly in their susceptibility to the coccidian infections; or, conversely, in the degree of resistance which they offer to the multiplication of these microorganisms inside their bodies. That one rat would produce about twelve times as many oocysts as another in the process of becoming immune is a somewhat amazing revelation. It is not altogether improbable that eventually rats will be found with a natural immunity that is approximately complete.

The promptness and completeness of the immunity which develops in coccidian infections of the rat is also impressive. Inoculations with 1,500 sporulated oocysts of *E. miyairii* and *E. separata* daily for five days, which was in all cases at least a day or two longer than was necessary, are sufficient to produce complete immunity after a patent period of five to eight days in the case of the former and of four to six days in the case of the latter.

In the passing of the weeks some of the immunity is lost. Our evidence is based upon four rats which showed the last coccidia on September 25. The next day they were fed a large number of viable oocysts, but they were still uninfected after the lapse of about two weeks. On December 12 each rat was fed 100,000 oocysts in a few drops of milk. On December 20, three of the rats commenced to discharge oocysts in moderate numbers and continued to do so for three days. In the feces of the fourth rat only one oocyst could be found, and that on December 21. A study of the rate of loss of immunity would be of extreme interest.

Immunity to *E. miyairii* does not render a rat immune to *E. separata*, and vice versa. Fourteen rats immunized to the former species were fed 1,500 oocysts of the latter for five successive days beginning the day after the last oocysts appeared. All of them became infected. Four rats immunized to *E. separata* were heavily infected after a single feeding of about 15,000 oocysts of *E. miyairii* given three days after the last oocysts of the

former species appeared. These experiments indicate that the one species of parasites does not completely immunize against the other, but do not of course, prove the absence of some degree of cross-immunity.

Is immunity in coccidiosis humoral or localized? This is a question which has never been answered. A solution of the problem was attempted by the use of parabiotic twins. The authors are indebted to Dr. E. Witschi and Mr. Robert T. Hill of the University of Iowa for demonstrating the technique of suturing the rats together. Our results with the operation were rather unsatisfactory, owing to the difficulty of preventing the sutures from becoming infected. Although there were many failures, four of the twins came through in a more or less satisfactory condition. In all cases the member of the pair on the right was a young rat which had just been completely immunized to *E. miyairii*, and the one on the left was a rat of similar age and weight which had never had coccidiosis. The case histories follow:

Pair 1. Suturing operation occurred on October 25. On the morning of November 21 the two rats together consumed 50,000 oocysts in about 20 cc. of milk. On the evening of November 25 the rat on the left was almost dead. Both rats were killed, and a post-mortem examination was made. The viscera of the rat on the right, previously immunized, were normal in appearance, and no sporozoites could be found in the scrapings of the wall of the small intestine. The lower half of the small intestine of the rat on the right was filled with a hemorrhagic exudate which contained myriads of motile merozoites.

Pair. 2. Suturing operation occurred on October 26. On November 21 the rat on the left was fed 1,500 sporulated oocysts of *E. miyairii* in milk, and on November 22 the two rats together consumed 50,000 more sporulated oocysts. On the morning of November 27 the rat on the left was dead, while the one on the right was still alive. At the post-mortem examination the intestine of the one on the right was apparently normal and the scrapings contained no sporozoites. The intestine of the one on the left was markedly hemorrhagic, and scrapings from the wall showed numerous sporozoites of coccidia and young oocysts.

Pair 3. The operation was performed on November 5. On November 21 each rat was fed 1,500 sporulated oocysts from a pipette. The infective feeding was repeated on November 22 and November 23. On the afternoon of November 28 oocysts commenced to appear in the feces of the rat on the left. They were present in large numbers on November 29 and 30 and December 1 and 2. During the morning of December 2 about 0.5 cc. of a one per cent methylene blue solution was injected intraperitoneally into the rat on the right to see if it would appear in the urine of the rat on the left, and thus attest to the expected exchange of blood between the two rats. Six hours later the injected rat was found dead. The cecum of the rat on the left still contained numerous oocysts. The rat on the right was negative for oocysts throughout the infection of its twin.

Pair 4. The operation occurred on November 6. The rats were infected on the same days and in the same manner as in the case of Pair 3. Oocysts were present in pellets from the left rat on November 28, 29, 30, December 1, 2 and 3. On December 3, the rat on the right was injected intraperitoneally with about one-fourth cc. of one per cent methylene blue solution. The next morning both rats were dead. The cecal content of the infected



rat contained a small number of oocysts. The rat on the right showed no oocysts at any time after the suturing operation.

The fecal material for the microscopic examinations of these rats was obtained by expressing the pellets from the anus.

The presence of dye in the cecal content of the rat on the left in Pair 4 supplied indisputable proof that there was an exchange of blood through the capillaries between the two members of the pair.

It is fully realized that the experiment with the parabiotic twins leaves much to be desired in respect to procedure and numbers of animals employed, but on the other hand it constitutes perhaps one of the strongest arguments that has yet been advanced against the humoral theory of immunity in coccidiosis. When and if a strain of rats which gives fairly consistent yields of oocysts is obtained, a more critical enumerative study will be carried on with the twins.

It is generally believed that coccidia do not multiply so well in older animals as in young ones. In order to test this out in a merely preliminary way, we procured from another department four male rats which were definitely known to be more than a year old. They were kept for more than three months in our laboratory before they were infected with coccidia. Thus these rats were at least fifteen months of age, and weighed between 350 and 400 grams apiece. It should be stated that the history of the rats as to the possible occurrence of previous infections with coccidia was not known. The animals were infected with 1,500 sporulated oocysts of *Eimeria miyairii* on five successive days. The total yields of oocysts from the rats during the subsequent patent period were, in round numbers, 13, 22, 32 and 38 millions, respectively. The series was not extensive enough to warrant any sweeping conclusions for or against an age immunity, but it does show clearly that older animals are capable of harboring coccidial infections of considerable intensity.

#### SUMMARY AND CONCLUSIONS

*Eimeria miyairii* and *E. separata* are two distinct micro-parasites of the rat. Measurements of oocysts of both species on each day throughout a number of infections show that each species has its own characteristic frequency distribution for length and breadth, as well as a shape index that is representative of it. In either pure or mixed infections an identification of the two forms may be made on the basis of oocyst size alone, although the factor of shape is a valuable adjunct in the case of mixed infections. Neither daily mean size nor shape index fluctuates widely throughout an infection, and there is certainly no tendency toward either larger or smaller oocysts as the infection progresses. In the case of *E. miyairii*, however, there were indications of a possible greater variability in oocyst size on the first day or two, as was shown by the standard deviations. The mean sizes and modes of frequency curves for oocysts taken from different rats throughout their entire infections, while showing slight differences, furnish little substantiation for claims that the individual host has a considerable influence upon the size of these forms. The experience of the authors shows that, in this case at least, great reliability can be placed upon the size and shape of the oocysts as a criterion of species.

There exists a definite, but not exceedingly high, correlation for the length and width of the oocysts of *E. miyairii*.



*Eimeria miyairii* and *E. separata* each demonstrates its own characteristic capacity to proliferate in the rat, as judged from the total oocyst yields throughout the entire infections. The former species shows by far the greater adaptability to the rat in this respect. Great as is the variability of total yields for *E. miyairii* in different rats, the lowest is at least four times the largest yield for *E. separata*.

Rats (at least in mixed strains) reveal a surprising heterogeneity in their suitability as hosts for coccidia. Of two rats receiving the same infective dose of sporulated oocysts, one may discharge many times as many oocysts as another. This phenomenon probably has a genetic background which is, however, still undemonstrated.

Infections in rats follow a more or less general course as regards daily oocyst production when produced by feeding a moderate number of sporulated oocysts on a number of, say five, successive days. In the case of *E. miyairii*, oocyst production begins on the seventh or eighth day after the first infective feeding, becomes heaviest on the ninth day, and gradually decreases in intensity until it ceases altogether after having occurred for from five to eight days. In the case of *E. separata*, the elimination of oocysts commences on the fifth or sixth day and continues for from three to five days thereafter. Upon the cessation of oocyst production the rat exhibits complete immunity to reinfection. Some of this immunity is lost in the passing of the weeks.

Some preliminary experiments with parabiotic twins have failed to show that a principle capable of protecting an animal to which it may be transferred exists in the blood of a rat in a state of immunity to *E. miyairii*.

The size and frequency of the dose, in the case of *E. miyairii*, has a definite effect upon the length of the patent period and the establishment of a state of complete immunity. A rat infected with a single light dose of sporulated oocysts (or even a moderately heavy dose as we have found in experiments not discussed here) does not discharge oocysts for quite so many days as one inoculated on successive days, and it rarely develops a complete immunity during such an infection.

The weight of the rat, within certain limits at least, has been shown to have no influence upon the yield of oocysts.

*E. miyairii* and *E. separata* are incapable of completing their development in mice or striped ground squirrels. The strong host-specificity exhibited by these two species adds strength to the general hypothesis that coccidia of the genus *Eimeria* are, with a few possible exceptions among bird forms, strictly host specific.

Other important differences between *E. miyairii* and *E. separata* (shown in table 1) are as follows:

1. The period required for the complete sporulation of the oocyst of the former is sixty-five to seventy-two hours at ordinary temperatures; for the latter, twenty-seven to thirty-six hours.
2. The oocysts of the former species are characteristically egg-shaped; those of the latter are ellipsoidal.
3. *E. miyairii* carries out its endogenous cycle principally in the epithelial cells of the lower part of the small intestine, though it may be found also in the large bowel. *E. separata* has not been found as yet in the small intestine, but it has been found in the cecum.

4. *E. miyairii* is pathogenic, at least to some rats, in large doses, especially when the inoculation is repeated on successive days. *E. separata* seems to be altogether harmless so far as external symptoms are concerned.

The technique of using standard doses and making daily counts of total number of oocysts eliminated promises to be a valuable one in the study of factors influencing coccidiosis in rats.

#### BIBLIOGRAPHY

BECKER, E. R., AND PHOEBE R. HALL

1931. *Eimeria separata*, a new species of coccidium from the Norway rat (*Epemys norvegicus*). Iowa State College Jour. Sci., 6:131.

DIEBEN, C. P. A.

1924. Over de morphologie en biologie van het rattencoccidium *Eimeria nieschulzi* n. sp. en zijne verspreiding in Nederland. Utrecht.

FISH, F.

1931. Quantitative and statistical analyses of infections with *Eimeria tenella* in the chicken. Am. Jour. Hyg., 14:560-576.

PÉRARD, CH.

1926. Sur la coccidiose du rat. Rec. Méd. Vét., Bul. et Mem. Soc. Méd. Vét., 102:120-124.

TYZZER, E. E.

1932. Criteria and methods in the investigation of avian coccidiosis. Science, 75:324-328.

A DISCUSSION OF SYNONYMY IN THE NOMENCLATURE OF  
CERTAIN INSECT FLAGELLATES, WITH THE DESCRIPTION OF  
A NEW FLAGELLATE FROM THE LARVAE OF LIGYRODES  
RELICTUS SAY (COLEOPTERA-SCARABAEIDAE)<sup>1</sup>

BERNARD V. TRAVIS<sup>2</sup>

*From the Entomology Section, Iowa Agricultural Experiment Station*

Accepted for publication April 5, 1932

Much of the early work in protozoology is very confusing, partly due to inadequate observations and descriptions, partly to the failure of authors to recognize the rules of priority, and partly to the absence of designated type species in the new genera. The identity of forms described only from unstained living specimens by the older workers is quite uncertain. For these reasons the synonymy of many species has become complex. The generic confusion involving *Monocercomonas*, *Retortamonas*, *Schedoacercomonas* and *Eutrichomastix* will be discussed in this paper, and suggestions regarding their validity will be given. Some of the difficulties of arriving at an accurate analysis of these names may be seen by a comparison of the viewpoints of different authorities in the groups.

MONOCERCOMONAS Grassi

Type—*coronellae* Grassi (1879) = *colubrorum* Hammerschmitt (1844) = *lacertae* Blochmann (1884).

Grassi (1879) created the genus *Monocercomonas* with the four subgenera *Monocercomonas*, *Retortamonas*, *Schedoacercomonas* and *Trichomonas*. The following new species were placed by him in the sub-genus *Monocercomonas*:

*M. hominis* Grassi = *Trichomonas hominis* Davaine (1860)

*M. caviae* Grassi = *T. caviae* Davaine (1875)

*M. coronellae* Grassi = *M. colubrorum* (Hammerschmidt) Grassi (1844)

*M. anatis* Grassi = (*pro parte*) *T. anatis* Kotlan (1923)

*M. batrachorum* Grassi = *T. batrachorum* Perty (1852)

*M. muris* Grassi = *T. muris* Grassi (1879)

*M. lacertae-viridis* Grassi = *Protermonas lacertae-viridis* Grassi (1879)

In 1881, Grassi removed all except *M. hominis* and *M. coronellae* from this sub-genus, but selected no type species. Later *M. hominis* was proved to be a *Trichomonas*, leaving only *M. coronellae*, which would then be the type. Grassi (1888) placed *Trichomastix* Blochmann in synonymy with his

<sup>1</sup>Journal Paper No. B47 of the Iowa Agricultural Experiment Station.

<sup>2</sup>The writer is greatly indebted to Dr. E. R. Becker for his assistance and criticism in the preparation of this paper and to Dr. C. J. Drake and Dr. H. H. Knight for their opinions concerning the complications of nomenclature.

*Monocercomonas*. Blochman (1884) described *Trichomastix lacertae*, and Dobell (1907) described *Trichomastix serpentis* from snakes. Dobell thought that *T. serpentis*, *M. colubrorum* and *M. coronellae* were the same species. Doflein (1916) regarded *M. coronellae* as a synonym of *M. colubrorum* and considered *T. serpentis* to be identical with these. Kofoid and Swezy (1915) followed Dobell, but did not mention the species described by Grassi. They believed that the *Eutrichomastix*<sup>3</sup> of snakes and lizards represented only one species. Wenyon (1926) ignored the parasites of snakes described by both Grassi and Hammerschmidt. Grassé (1926), who has studied reptilian flagellates, points out: "L'espèce type des *Monocercomonas* devrait donc s'appeler *Monocercomonas colubrorum* (Hammerschmidt, 1844), le genre *Eutrichomastix* disparaissant de la nomenclature." He does not, however, make the indicated change. He recognizes *E. colubrorum* Hammerschmidt, placing *E. coronellae* Grassi, *E. lacertae* Blochmann, *E. viperæ* Leger, *E. serpentis* Dobell, *E. mabuia* Dobell and *E. saurii* Fonseca in synonymy with this species. Reichenow (1929) follows Grassé's work on this genus.

Stiles (1902) selected *E. lacertae* as the type of *Eutrichomastix* and *M. coronellae* as the type of *Monocercomonas*. These flagellates apparently belong to the same genus, with *Monocercomonas* having priority. Hence, *Monocercomonas* should replace the genus *Eutrichomastix*.

This genus contains the following species, which are all new combinations except for *colubrorum*: *Monocercomonas colubrorum* (Hammerschmidt) Grassi, *M. batrachorum* (Dobell), *M. ruminantium* (Braune), *M. gallinarum* (Martin and Robertson), *M. caviae* (Grassi), the doubtful variety *M. caviae* var. *rossica* (Yakimoff, Wassilevsky, Korniloff and Zwietskoff), *M. motellae* (Alexeieff), *M. salpae* (Alexeieff), *M. trichopterae* (MacKinnon), *M. passali* (Tanabe), *M. aguti* (Cunha and Muniz), *M. termitis* (Bernstein), *M. phyllophagae* (Travis and Becker), *M. cuniculi* (Tanabe), *M. coprocola* (Alexeieff), *M. rhinocrici* (Fonseca), *M. cruzi* (Cunha and Muniz), *M. globosis* (Cunha and Muniz) and *M. gracilis* (Cunha and Muniz).

#### MONOCERCOMONOIDES n. nom.

Type—*melolonthae* Grassi (1879).

Grassi (1879) placed one species in his sub-genus *Retortamonas*, *R. gryllotalpae*. The synonymy of this species was further complicated by him in 1881, when he placed *gryllotalpae* in the genus *Plagiomonas*.

In 1879 he placed the following new species in the sub-genus *Schedoacercomonas*:

*S. gryllotalpae* Grassi = *Monocercomonas insectorum* Grassi

*S. melolonthae* Grassi (1881)

*S. caviae* Grassi = *nomen nudum*

*S. muscae-domesticae* Grassi = *Herpetomonas muscae-domesticae* Stein (1878)

<sup>3</sup>This generic name was created by Kofoid and Swezy (1915) as a substitute for the *Trichomastix* Blochman (1884) of protozoologists since Vollenhoven (1878) had already given this name to an insect.



Grassi (1879) described this sub-genus with three words, "quasi senza coda," but he gave no description of the species at this time. In 1881, he called *S. gryllotalpae* and *S. melolonthae*, *Monocercomonas insectorum*, but this can not hold since he had already applied this generic name to another group of flagellates. Stiles (1902) designated *S. caviae* as the genotype, but this can not be accepted since this species was undescribed. Wenyon (1926) considered *S. gryllotalpae* and *R. gryllotalpae* to be synonymous, with *R. gryllotalpae* having priority. He then wrote, ". . . . *Schedoacercomonas gryllotalpae* and *S. melolonthae*, both of which appear to belong to the same genus, the correct name for these flagellates is *Retortamonas*, and not *Monocercomonas*." Grassé (1926) recognized *R. gryllotalpae* as a distinct species when he said, "Mais dans la Courtilière nous n'avons trouvé jusque-ici que deux Flagellés: *Retortamonas gryllotalpae* Grassi (1879) et un *Hexamastix*." Grassé designated the type species as *S. gryllotalpae*, which he has proved to be *Hexamastix Alexeieff*.

Wenrich, in an unpublished paper read at the 1931 A. A. A. S. meetings held in New Orleans, has definitely proved *R. gryllotalpae* Grassi (1879) to be cogenetic with the various species of *Embadomonas* Mackinnon (1911), so the "*Monocercomonas*" of insects can not be called *Retortamonas*. Grassé (1926) removed the genotype of *Schedoacercomonas* to the genus *Hexamastix*, a move which necessitates a new name for Grassi's *S. melolonthae*. *Monocercomonoides* is here proposed as a new generic name for these organisms with *M. melolonthae* as the type species.

The genus contains the following species, which are new combinations: *Monocercomonoides melolonthae* (Grassi), *M. orthopterorum* (Parisi), *M. melolonthae* var. *tipulae* (Grassé), *M. cetoniae* (Jollos) and *M. hassalli* (Cunha and Muniz).

#### HEXAMASTIX CONFUSA n. nom.

Grassi (1879) divided his new genus *Monocercomonas* into four subgenera, namely: *Monocercomonas*, *Trichomonas*, *Retortamonas*, and *Schedoacercomonas*. Unfortunately, he described two *gryllotalpae* species in this genus, *R. gryllotalpae* and *S. gryllotalpae*. Both were described in the same paper with *R. gryllotalpae* having line precedence. Hence the species *S. gryllotalpae* becomes a homonym and must be discarded. Grassé (1926) removed this species to the genus *Hexamastix* Alexeieff. *Hexamastix confusa* is proposed as a new name for this organism.

#### MONOCEROMONOIDES LIGRODIS n. sp.

During the past two years (1930-32) the writer has been engaged in a study of white grubs (*Phyllophaga* spp.) in Iowa. A preliminary paper on the protozoan fauna of these insects was published by Travis and Becker (1931). In the summer of 1931, large numbers of *Ligyrodes* larvae were found under old rotten straw stacks and manure piles near Leon, Iowa. These grubs were similar to *Phyllophaga* larvae and are often mistaken for them. Several of the grubs were brought to the laboratory at Ames for an investigation of their intestinal inhabitants.

Examinations of their rectal material revealed the presence of myriads of protozoan parasites belonging to the class *Mastigophora*. All individuals collected from decaying straw contained protozoa, whereas those from cow

dung were uninfected. The protista were studied both in the living state and in permanent slides stained with Heidenhain's iron-hematoxylin. They are, apparently, harmless commensals feeding only upon bacteria.

The body varies from round (Fig. 4) to ovoidal (Fig. 1) in shape. The pellicle is thin and easily distorted. The protoplasm stains slightly with hematoxylin. Bacteria are contained in the food vacuoles, although no cytostome is observable.

Flagella vary from four (Fig. 1) to ten (Fig. 5) in number, depending upon the state of division of the animal; typically, four arise in pairs from individual blepharoplasts (Fig. 8). These blepharoplasts are very small and are usually grouped in pairs. They are near the anterior sides of the nucleus, and are often so closely pressed into the nuclear wall that they are indistinguishable from the chromatin material (Fig. 6). No rhizoplast connects the basal granules or passes from them to the nucleus as Grassé (1926) has described in this genus.

A filamentous axostyle arises from one of the blepharoplasts, curves towards the periplast and terminates at the caudal end of the body (Fig. 1). This structure is not observable in all specimens, but binucleated forms often possess two of them. Enlargements are frequently seen at the posterior end of these filaments where they can touch the pellicle (Fig. 9).

The nucleus is the most distinguishing character of the species. This spherical organelle, located at the extreme anterior end of the body, has an average size of  $2.39\ \mu$  in uninucleated and  $1.98\ \mu$  in binucleated specimens. Sixty per cent of the individuals are binucleated (Fig. 9), but uninucleated forms are considered typical (Fig. 1). The nucleus is surrounded by a thin but indistinct membrane beaded on the inside with chromatin granules. The karyosome is small and typically central in position, and surrounded by a narrow halo (Fig. 1). Free chromatin granules occur between this clear area and the peripheral layer of chromatin. Large siderophilous granules are scattered in the cytoplasm (Fig. 6). The karyosome and nucleus are relatively much smaller in this species than in other representatives of *Monocercomonoides*.

The darkly staining karyosome divides during nuclear division, and then becomes indistinguishable from the other chromatin granules. The two mitotic figures observed were in binucleated individuals (Figs. 2, 3). Six specimens with four nuclei (Figs. 4, 5) and one with five nuclei were seen. Frequently individuals contain one small and one large nucleus, and in such cases there are no flagella near the smaller nucleus (Fig. 6). This phenomenon seems to have been the result of unequal nuclear division.

No cysts were present. The size of the trophozoite varies from  $4.7\ \mu \times 4.75\ \mu$  to  $11.4\ \mu \times 9.88\ \mu$ . The average size for twenty specimens is  $9.4\ \mu \times 8.2\ \mu$ .

#### SPHAERITA sp.

The cytoplasm of *Monocercomonoides ligyrodis* is so heavily infected with *Sphaerita* parasites that it is not uncommon to see three or four plasmodia within the body of a single individual. The spores of this fungus are of two kinds—small and large.

The small spores measure about  $0.9\ \mu$  in diameter. They stain lightly with hematoxylin and are surrounded by a thin cell membrane. The plasmodia vary from about  $1.8\ \mu$  to  $5.7\ \mu$  in diameter and are almost filled with morula-shaped masses of spores.

The large spores measure about  $1.5\mu$  in diameter. They have a thick spore wall that stains heavily with hematoxylin. The plasmodia vary from about  $2.3\mu$  to  $6.2\mu$  in diameter. These large spores are scattered in the plasmodium and are not so numerous as the small ones.

It is quite possible that these two types of spores are merely different stages of the same species, but a series of individuals demonstrating this can not be found in the available material. Also, the young stages which are so important in the determination of species were not seen. The writer has chosen not to give this *Sphaerita* a specific name until a better series of specimens is obtained.

## LITERATURE CITED

## BLOCHMANN

1884. Bemerkungen über einige Flagellaten. Zeitsch. f. wiss. zoo. 40:42-47.

## DOBELL, C.

1907. *Trichomastix serpentis* n. sp. Quart. Jour. Micr. Sci., 51:449-458.

## DOFLEIN, F.

1916. Lehrbuch der Protozoenkunde. Vierte Auflage. Jena.

## GRASSÉ, P. P.

1926. Contribution à l'Etude des Flagellatés parasites. Arch. Zool. Expt. et Générale, 65:345-602.

## GRASSI, B.

1879. Die protozoi parassiti e specialmente di quelli che sono nell'uomo. Gazz. Med. Ital. Lomb., 39:445-448.

---

1881. Intorno ad alcuni protisti endoparassitici. Attid. Soc. Ital. Sc. Nat., 24:133-224.

---

1888. Parasitic Protozoa. Jour. Royal Micr. Soc., Pt. 2:975 (Abstract).

## HAMMERSCHMIDT, C. E.

1844. Neues Entozoon in Darm der Schlangen. Heller's. Arch. f. Phys. u. path. Chem. und. Mikrosk. 83. (Original not seen.)

## KOFOLD, C. A., AND O. SWEZY

1915. Mitosis and multiple fission in trichomonad flagellates. Proc. Amer. Acad. Arts and Sci., 51:289-378.

## REICHENOW, E.

1929. Lehrbuch der Protozoenkunde (Revision of Doflein) Fünfte auflage. Jena.

## STILES, C. W.

1902. The type species of certain genera of parasitic flagellates, particularly Grassi's genera of 1879 and 81. Zool. Anz., 25:689-695.

## TRAVIS, B. V., AND E. R. BECKER

1931. A preliminary report on intestinal protozoa of white grubs (*Phyllophaga* spp. -Coleoptera). Iowa State College Jour. Sci., 5:223-235.

## WENRICH, D. H.

1931. *Retortamonas gryllotalpae* Grassi 1879, and its relation to the genus *Embadomonas*. Anatomical Record 51, No. 1, supp.:66-67 (abstract).

## WENYON, C. M.

1926. Protozoology. London.

PLATE I  
DESCRIPTION OF FIGURES

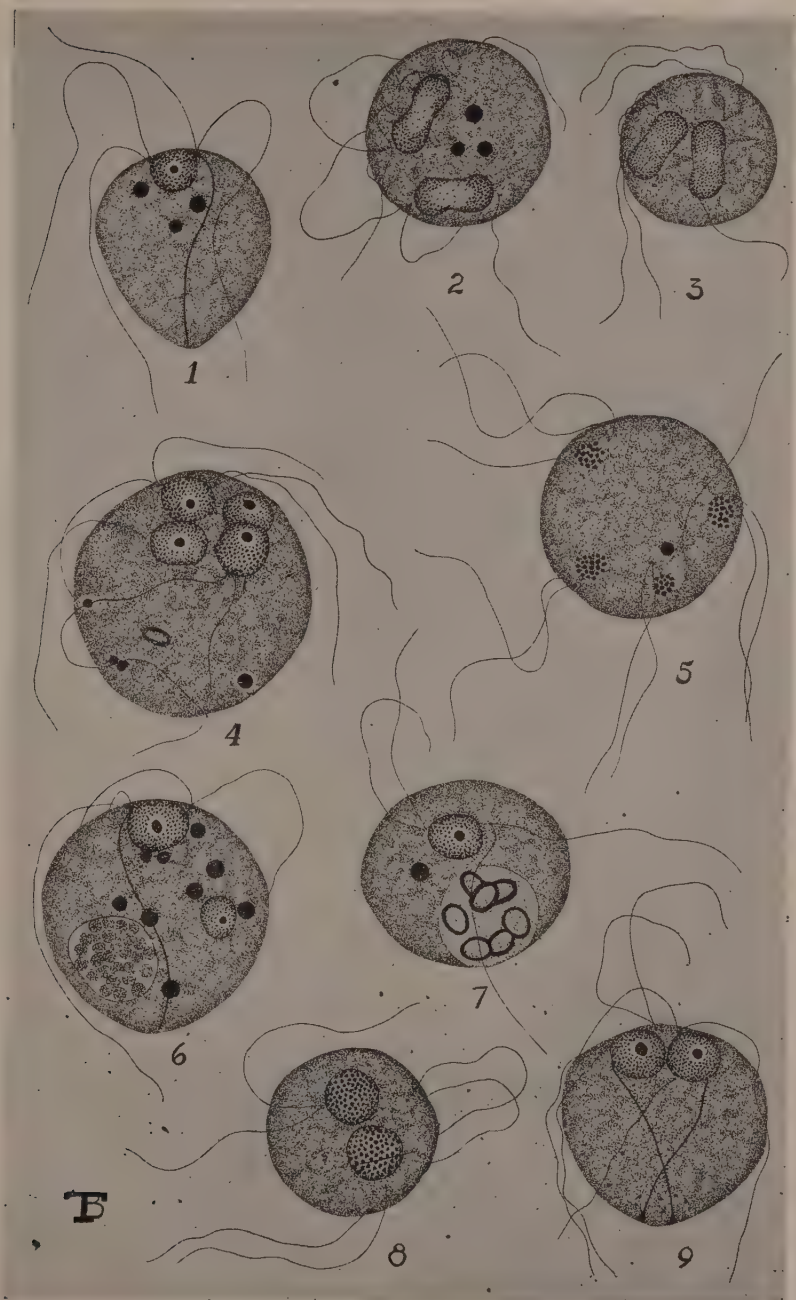
x 3000

*Monocercomonoides ligyrodia* n. sp.

- Fig. 1. Typical trophozoite.
- Fig. 2. Mitotic figure with chromatin granules collected at ends of nuclei.
- Fig. 3. Mitotic figures with scattered chromatin granules.
- Fig. 4. Trophozoite with four well developed nuclei.
- Fig. 5. Trophozoite with four unorganized nuclei.
- Fig. 6. Trophozoite with one large and one small nucleus; cytoplasm containing small Sphaerita spores.
- Fig. 7. Trophozoite with large Sphaerita spores.
- Fig. 8. Binucleated individual illustrating flagella arising from separate granules. Chromatin in the form of small granules with indistinguishable karyosome.
- Fig. 9. Binucleated individual with two axostyles. Axostyles enlarged at the posterior end.



## PLATE I





# ON THE COAGULATION OF BLOOD FROM THE COCKROACH, *PERIPLANETA ORIENTALIS* (LINN.), WITH SPECIAL REFERENCE TO BLOOD SMEARS

J. FRANKLIN YEAGER, W. EARL SHULL AND MILTON D. FARRAR

*From the Department of Zoology and Entomology, Iowa State College*

Accepted for publication April 14, 1932

This paper is a report of studies upon (a) the nature of the coagulation process in cockroach blood and (b) the preparation of cockroach blood smears, the cells of which are randomly distributed. In order to obtain satisfactory blood smears it has been found necessary to prevent the occurrence of coagulation. All of the observations and experiments presented in the following paragraphs have been made on blood from the roach *Periplaneta orientalis* (Linn.); enough additional work has been done upon blood from *Periplaneta fuliginosa* Serville to indicate that blood coagulation is essentially the same in both species<sup>1</sup>.

The investigations of Leo Loeb (16), Tait (22, 23), Tait and Gunn (24), Geddes (4), Hardy (9), Haliburton (8) and others have shown that blood cells play an important or even predominant role in the blood coagulation of arthropods, especially of the crustacea and of the arachnid, *Limulus*. The work of Loeb upon crustacean and *Limulus* blood coagulation is of special interest from the standpoint of this paper. According to Tait (22), the coagulation of crustacean blood can be arranged in three roughly separable categories: (a) coagulation involving only the formation of a coagulum of blood cells; (b) coagulation involving only the formation of a coagulum by constituents of plasma or serum; and (c) coagulation involving both of these processes. It has been shown by Hardy (9), Tait (23, 24) and others that, in at least some crustacea, plasma coagulation is initiated in the region of and apparently by certain unstable blood cells that show disintegrative changes just previous to plasma coagulation. It has also been shown, especially by the work of Loeb, that cell coagulation and plasma coagulation are distinctly different processes in the same crustacean blood; the former involves changes in the physico-chemical properties of the blood cells, while the latter apparently involves fibrin formation by plasma or serum constituents. This work has also shown that certain factors (as, for example, potassium oxalate) may inhibit plasma coagulation without inhibiting cell coagulation.

Similar studies on insect blood are relatively few. Many reports of experimental investigations of insect blood either do not mention coagulation or merely state incidentally that it does or does not occur. Among earlier workers, Geddes (4) studied blood coagulation in a number of different invertebrates and concluded "that the clot which appears in any invertebrate corpuscular fluid is formed, always partly, and sometimes wholly, by the fusion of the finely granular amoeboid corpuscles therein

---

<sup>1</sup>Dr. H. H. Knight, of Iowa State College, has very kindly identified the two species of cockroach.

suspended." He considers the clot, which he calls a plasmodium, to be a syncytial coalescence of the blood cells and concludes further "that the power of coalescing with its fellows, under favorable circumstances, to form a plasmodium, is at any rate a very widely-spread if not a general property of the amoeboid cell." Poulton (21), on the other hand, working with various larval and pupal bloods, describes the ultimate coagulum as a solid black clot that is due to oxidation; he does not describe the respective roles of cells and plasma in the formation of the coagulum. Griffiths (6) confirms observations made by Poulton that insect blood clots after a variable length of time, that in some "samples" clotting does not occur at all and that larval blood coagulates more rapidly than pupal blood. Absence of coagulation is reported by Bishop, Briggs and Ronzoni (2) for larval honey bee blood and by Barratt and Arnold (1) for the bloods of *Hydrophilus piceus* and *Dytiscus marginalis*; the latter used smears. Geyer (5) merely states that he depended upon blood coagulation to close up the wounds due to castration operations on various insects. Cuenot (3) finds fibrin in the blood of arthropods generally, but states that its occurrence in insects is not constant, appearing less plentifully in some species than in others. This author apparently considers the blood clot of most invertebrates, except in the echinoderms, to be exclusively of plasma origin. Landois (14) states that it is possible to recognize microscopically the fibrin of fresh blood as a network that contains blood corpuscles and that gradually contracts. Tait (23), in a consideration of amoeboid movement, states that cockroach blood contains non-amoeboid "hyaline thigmocytes" and amoeboid "granular thigmocytes." Muttkowski (19) reports the formation of both cell and plasma coagula in insect blood, the latter consisting of fibrin fibers. Haber (7) similarly reports the occurrence of cell and plasma (fibrin) coagula in the blood of *Blatella germanica*. Paillot (20), recognizing the undesirability of blood coagulation in certain types of experimental work, describes an adaptation of Doyon's method to insects; he states that the injection of nucleic acid (extract of beef mesenteric ganglion) into an insect prevents coagulation of the animal's blood. Yeager and Tauber (25) found blood coagulation to be a serious interference in the making of total blood cell counts.

A survey of the available literature on the subject of insect blood coagulation is apt to leave one somewhat confused as to details of the changes taking place during the clotting process, as well as to its actual occurrence in various insect species. It is hoped that the following observations and experiments will serve to elucidate somewhat the details of the coagulation process in cockroach blood.

The animals used were obtained from Mississippi (*P. orientalis*) and from Iowa (*P. fuliginosa*). Previous to being used they were kept in specially constructed wire cages under conditions of room temperature and humidity and were well fed. Only large nymphs and adults were used.

#### METHODS

(a) *The Coagulation Process.* The following simple methods were utilized in studying the coagulation process. The blood was always obtained from the severed end of an antenna, gentle pressure being applied with the fingers to the animal's body when necessary.



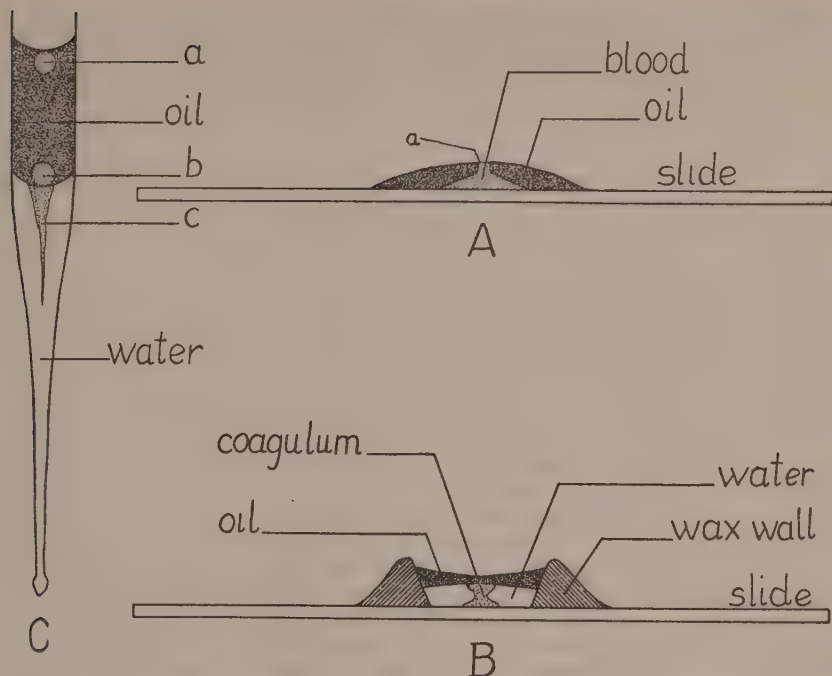


Fig. 1. Fresh preparations of insect blood: A. Drop of blood immersed in a drop of oil (Nujol) on glass slide; exposed surface of blood at *a* not yet covered over by oil. B. Wax chamber containing a layer of oil on a layer of water; the blood coagulum is shown hanging from the interface. C. Micropipette, sealed at end, containing layer of oil on layer of water; *a* and *b* represent successive positions of a drop of blood placed in the oil; *c* represents the blood coagulum hanging from the interface.

*Fresh Preparations.* When evaporation was desired, a drop of blood was placed on a clean glass slide and observed immediately. When evaporation was not desirable (10), the blood was placed in a larger drop of Nujol on a slide, the Nujol preventing evaporation and allowing microscopic observation. This is illustrated in figure 1, A; the blood is represented as having settled and partially spread upon the slide, without having yet become completely covered over by the oil. Observations of whole blood and plasma from experimental and control animals were made in this way. The photographs of Plate I and figures 6, 7, 8 and 9, Plate II, are of this type of preparation.

Macroscopic observations of whole blood or plasma in an oil-water system is illustrated by figure 1, C. A drop of blood is placed in the oil at *a*, settles to the surface at *b* and, after a variable length of time, undergoes a sudden reaction (probably involving the wetting and spreading of the blood drop at the oil-water interface) as a result of which the plasma or serum is precipitated into the water phase while the blood clot remains spread out on or hanging from the oil-water surface. The systems used

consist of Nujol-distilled water or Nujol-Hobson solution<sup>2</sup>. By means of the adaptation shown in figure 1, B, microscopic observations of whole blood or plasma at the interface can be readily made.

Blood smears of completely clotted, partially clotted and unclotted blood were studied in addition to fresh preparations; see Plate II, figures 10, 11, 12, and Plate III.

(b) *The Preparation of Blood Smears.* When fixed and stained blood smears are made from the blood of normal, untreated animals, they show many of the cells to be agglutinated (Plate III, Figs. 13, 14, 15, 16, 17.) Although such smears illustrate coagulum formation and, to some extent, may be used to determine cell types, they are not suitable for obtaining differential cell counts. Rapid working alone does not suffice, as in the case of vertebrate blood, for the preparation of satisfactory smears. Loeb (15) states that in the blood of *Limulus*, in which only the cells and not the plasma contribute to the formation of a coagulum, coagulation can be prevented by collecting the blood in warm water. This suggestion has been adapted to the insect and the following procedure found to inhibit coagulation in the cockroach without the necessity of diluting the blood. The animal is immersed in water at 60° C. for ten minutes, removed and wiped dry. A drop of blood from the severed end of an antenna is touched to the surface of a clean glass slide and the smear made with the paraffin coated end of another slide. The cells of the resulting smear are evenly distributed and give little or no indication of coagulation (Pl. II, Fig. 11). The smears were dried in the air and stained with Delafield's hematoxylin and eosin or with Wright's blood stain. Best differential staining was obtained with Wright's stain that had been subjected to reflux distillation for several hours. Permanent preparations were cleared in xylol, mounted in balsam and covered with a cover-slip. The optimum time of staining with Wright's was found to be about 1.6 minutes with concentrated stain and about 8 minutes with the stain diluted.

## RESULTS

(a) *The Coagulation Process.* Microscopic observations of a drop of *P. orientalis* blood in oil on a glass slide indicate that coagulation involves extensive changes in the blood cells and little visible alteration in the blood plasma. In general a granular precipitate, the particles of which exhibit Brownian movement (Pl. I, Fig. 5, a), eventually appears in the plasma, but the latter does not form a visible coagulum and apparently undergoes no great change in viscosity. The coagulation process begins while the blood is being collected. Many of the cells that escape with the plasma from a cut antenna appear to have flattened fusiform shapes (Pl. II, Fig. 9, a). Some of the cells can be seen stuck to the antenna, when the latter is immersed in the drop of blood being collected from it, and are removed along with the antenna from the blood. Many of the remaining cells undergo a change in form by quickly losing their spindle-like shapes and rounding up into spheroids or ovoids (Pl. I, Fig. 2, a; Pl. II, Fig. 7). Some few cells seem to retain their spindle or cigar-like forms. For purposes of descrip-

---

<sup>2</sup>See reference (11). The solution consists of 0.161 M NaCl, 0.003 M KCl and 0.002 M CaCl<sub>2</sub>.

tion the cells that round up may be roughly divided into two groups; it should be emphasized, however, that this classification is made merely for descriptive purposes and may or may not represent real cell types.

(1) The cells of the first group round up and become so refractive that it is difficult to distinguish nucleus and cytoplasm (Pl. I, Fig. 2; Pl. III, Fig. 13, and Fig. 15, a). They develop thread pseudopodia (Pl. I, Fig. 3, a, and Fig. 4, a) and gradually agglutinate, forming cell clumps (Pl. I, Fig. 2, b, c). Following agglutination, the clumps of cells soon become transformed into granular masses (Pl. I, Fig. 3, b, and Fig. 4, b) that contain a number of round bodies (Pl. I, Figs. 4, c, 5, c; Pl. II, Fig. 8, a, b); the latter appear to be either cells or nuclei. At times, spherical droplets of what might be oily material appear in the clumps of cells; these are of varying size and number and are similar to the droplets shown in figures 2, 3 and 4 (Pl. I, d); figure 8, c (Pl. II); and figure 18, c (Pl. III). From this stage on, but especially later, the clumps of cells are seen to be connected with each other by long and short thread pseudopodia (Pl. I, Fig. 3, a) and by broader bands of fibrous, granular material, apparently consisting of elongated, disintegrated cells (Pl. II, Fig. 8, d). Such a coagulum or network of fibrous material can be seen both in the fresh preparation (Pl. I, Fig. 4; Pl. II, Fig. 8) and on the stained slide; the fibrous and cellular nature of the coagulum is demonstrated better by Delafield's hematoxylin and eosin (Pl. III, Figs. 14, 16) than by Wright's stain (Pl. III, Figs. 13, 15). The granular precipitate (Pl. I, Fig. 5, a; Pl. II, Fig. 8, e) appears first in the clumps of agglutinated cells and later in the interclump spaces. It is not impossible that this plasma precipitate is formed due to the liberation of some material from the agglutinated cells.

(2) The cells of the second group usually appear much less refractive than those of the first group; they possess much less conspicuous cell boundaries and the nuclei appear as ovoid or spheroid bodies of distinct outline surrounded by a relatively less distinct cytoplasm in which fairly large refractive droplets or granules are visible. These cytoplasmic granules show Brownian movement, indicating a fluidity of the cytoplasm. Eventually, many of these cells appear to become connected with other cells (usually those of the clumps) by means of fibers and later disintegrate, the nucleus becoming more indistinct and the whole cell mass taking on a granular, precipitated appearance. The cytoplasmic granules now show no Brownian movement, although granules close to but not included in the disintegrated cell mass exhibit active Brownian movement, indicating the viscosity of the cell mass to be high and that of the surrounding medium low. Some of these cells appear to maintain their integrity for relatively long times. These cells appear similar in form to certain cells (Pl. III, Fig. 15, b) seen in smears of coagulated or partially coagulated blood stained with Wright's stain.

After eight or ten hours, the clumps of agglutinated cells appear more compact (Pl. II, Fig. 8, f) and are connected with each other by broad bands of cell material (Pl. II, Fig. 8, d) and by thread pseudopodia, while the interclump spaces seem larger, may still contain a few isolated cells and a granular precipitate (Pl. I, Fig. 5, a; Pl. II, Fig. 8, e).

In similar fresh preparations of blood from animals heated at 60° C. for 10 minutes, the blood cells (Pl. II, Fig. 6) are not rounded up, are not

agglutinated and give no evidence of coagulum formation, even when agitated with a needle, but seem to retain their natural forms; many of the cells contain refractive granules or droplets of various sizes.

Coagulation can be partially prevented (Pl. II, Fig. 7; Pl. III, Fig. 17) by heating the animal at relatively low temperatures for ten minutes or by heating them for shorter times at higher temperatures; whether or not the product of time and temperature is constant or variable has not been determined. Table 1 shows the effect of heating animals for ten minutes at various temperatures.

TABLE 1. *Effects upon cockroach blood coagulation of heating animals for 10 minutes at the given temperatures; observations made upon fresh preparations and stained smears*

Animal No.	Temperature	Fresh preparation	Slide No.	Smear	Stain
1	Room	Coagulation	137	Coagulation	Wright's
2	"	"	138	"	"
3	"	"	139	"	Delafield's
4	30° C.	"	140	"	Wright's
5	"	"	141	"	"
6	"	"	142	"	Delafield's
7	35° C.	"	143	"	Wright's
8	"	"	144	"	"
9	"	"	145	"	Delafield's
12	40° C.	Partial coagulation	148	Partial coagulation	Wright's
13	"	"	149	"	"
14	"	Coagulation	150	Coagulation	Delafield's
15	45° C.	Partial coagulation	151	Partial coagulation	Wright's
16	"	"	152	"	"
17	"	"	153	"	Delafield's
18	50° C.	No coagulation	154	Slight coagulation	Wright's
19	"	"	155	"	"
20	"	"	156	"	Delafield's
21	55° C.	"	157	No coagulation	Wright's
22	"	"	158	"	"
23	"	"	159	Slight coagulation	Delafield's
24	60° C.	"	160	No coagulation	Wright's
25	"	"	161	"	"
26	"	"	162	"	Delafield's
27	65° C.	No coagulation; plasma precipitate	164	? (few cells)	Wright's
28	"	No coagulation; plasma precipitate	165	? (few cells)	"
29	"	No coagulation; plasma precipitate	166	Slight coagulation	Delafield's
30	70° C.	No blood obtained	—	—	—
31	"	"	—	—	—
32	"	"	—	—	—

The foregoing table shows that as the temperature increases there occurs first a partial inhibition of coagulation; the blood cells round up, become more refractive and agglutinate, but do not disintegrate, even upon standing for several hours (Pl. II, Fig. 7). At a higher temperature, coagulation



is completely inhibited and the plasma remains non-granular (Pl. II, Fig. 6), while at a still higher temperature a granular precipitate, in active Brownian movement, appears in the plasma along with the uncoagulated cells. At a still higher temperature, the blood apparently gels in the animal's body and so can not be obtained for observation. The table also indicates that evidences of coagulation disappear at a somewhat lower temperature in the fresh preparation than in the blood smear, where mechanical agitation and evaporation may combine to produce this difference.

The cellular nature of the coagulum lends itself to ready microscopic demonstration when a drop of blood is placed in the oil-water system illustrated in figure 1, C. Whole blood and filtered blood from unheated and heated animals were placed in systems formed of Nujol-distilled water and of Nujol-Hobson solution. The observations are recorded in table 2.

TABLE 2. *Observations of whole and filtered cockroach blood from heated and unheated animals and placed in a Nujol-water system*

Animal heated to	Condition of blood	Cell coagulum		In aqueous phase	Aqueous phase
		At interface	Hang-from interface		
Unheated	Whole blood	Yes	Yes	Plasma ppt.	Dist. H <sub>2</sub> O
"	Filtered blood	No	No	" "	" "
"	Whole blood	Yes	Yes	No plasma ppt.	Hobson's sol.
"	Filtered blood	No	No	" " "	" "
60° C., 10 min.	Whole blood	No	No	Plasma ppt., unclotted cells	Dist. H <sub>2</sub> O
"	Filtered blood			Plasma ppt.	" "
"	Whole blood	No	No	No plasma ppt., unclotted cells	Hobson's sol.
"	Filtered blood	No	No	No plasma ppt., unclotted cells	" "

The results given in this table show that coagulum formation is dependent upon the presence of unstable blood cells and that no visible fiber formation occurs in filtered blood from either heated or unheated animals. The experiment does not exclude the possibility that a fibrous plasma coagulum may have been removed along with the cell coagulum during filtration. In no case, however, has observation of a fresh preparation indicated the presence of a fibrous coagulum in the blood plasma or serum, nor has it shown that the granular plasma precipitate of either undiluted or diluted blood becomes transformed into fibers.

Further evidence of the essential difference in the coagulation phenomena of vertebrate and cockroach blood is yielded by an experiment<sup>3</sup>, the results of which are given in table 3.

This experiment shows that the anticoagulant, potassium oxalate, effective in mammalian blood, is without effect when injected into the cock-

<sup>3</sup>Mr. Oscar E. Tauber very kindly aided in this and other injection experiments.

TABLE 3. *Effect of injections of (a) Hobson's solution and (b) 33 per cent K oxalate in Hobson's solution upon coagulation of cockroach blood*

Animal No.	Body weight in grams	Fluid injected	Amt. fluid injected		Fresh preparation*	Animal four hours later
			% by Wt.	cc.		
1	0.2026	Hobson's sol.	1	0.0020	Blood coagulated	Normal-active
2	0.1617	" "	2	0.0032	" "	" "
3	0.1700	" "	4	0.0068	" "	" "
4	0.1058	" "	8	0.0084	" "	" "
5	0.1259	" "	10	0.0130	" "	" "
6	0.1965	" "	20	0.0393	" "	" "
7	0.1685	33% K oxalate in Hobson's sol.	1	0.0017	" "	On back-quiet
8	0.1373	" "	2	0.0027	" "	" "
9	0.1197	" "	4	0.0048	" "	" "
10	0.1911	" "	8	0.0153	" "	On back-dead
11	0.1209	" "	10	0.0121	" "	" "
12	0.2169	" "	20	0.0434	" "	" "

\*Blood examined in oil on slide approximately 30 minutes after injection.

roach, even when the injected fluid is fatal to the animal (due probably to reduction in concentration of soluble calcium salts in the animal). It has also been found that collection of a drop of roach blood in a larger drop of Hobson's solution containing 33 per cent potassium oxalate does not prevent coagulation.

(b) *The Cell Coagulum.* Some idea of the properties of the cell coagulum described above can be obtained by observing microscopically and at the same time agitating the coagulum with the point of a needle. Under these conditions the coagulum appears to have the properties of stickyness, elasticity and, possibly, contractility. (1) Stickyness: the cells of the clot stick to the slide, to the needle and especially to each other. It is possible to remove nearly all of the cells from the plasma (or serum) as a single cell mass stuck to the needle point. In uncoagulated blood from heated animals, the cells do not stick to slide, needle or each other under the same conditions. (2) Elasticity: when a portion of the coagulum is stuck at different points to slide and needle and is stretched to the breaking point, the broken portions tend to return to their initial lengths. (3) Contractility: it has been noted above that upon long standing the clumps of agglutinated cells appear to become more compact. In the case of *Limulus* blood, such behavior has been taken by Loeb (15) as evidence of contraction of the fibrous bands of the coagulum, somewhat as known to occur in the case of the fibrin coagulum of mammalian blood. It is of interest, in this connection, that the following occurrence has been noted a number of times in early stages of cockroach blood coagulation: a single cell may be seen to suddenly and rapidly move, as though jerked in the direction of its movement; this type of movement would result in the case of a cell, adhering to the surface of the glass slide and acted upon by an elastic fiber, when

the force of elasticity becomes greater than the force of adhesion. The behavior of the surrounding cells together with the peculiar movement of the cell observed renders the probability slight that this movement is due to plasma or serum currents overcoming the adhesion of the cell to the slide.

(c) *Blood Smears*. Blood smears with randomly distributed cells have been prepared by the heating method described above. Comparison of smears from unheated (Pl. III, Fig. 13) and from heated (Pl. II, Fig. 11) animals shows the superiority of the latter in cell distribution. No attempt has been made in the present work to identify cell types relative to the classifications of Hollande (12) and others, although cursory observations indicate that there are probably at least three different blood cell types in *P. orientalis*: (1) the cell shown in Plate II, figure 9, b and figure 12, a; Plate III, figure 18, a and figure 19. This cell possesses a coarsely granular, basophilic nucleus and a relatively large amount of cytoplasm, which usually appears non-granular when stained with Wright's, but which, especially in the case of blood showing partial or complete coagulation, may contain large, eosinophilic granules (Pl. II, Fig. 10, a; Pl. III, Fig. 19). (2) The cell shown in Plate II, figure 12, b; Plate III, figures 15 and 18, b. This cell often displays a more compact nucleus than the above mentioned type. Its nucleus, stained with Wright's in partially or completely coagulated blood smears, usually exhibits a decided eosinophilia. (3) A very small cell, equivalent in appearance and relative size to one of the daughters of the dividing cell shown in Plate III, figure 20 and Plate II, figure 11, a; this cell has a markedly compact, decidedly basophilic nucleus, surrounded by a very thin rim of cytoplasm. It may correspond to Hollande's (13) proleucocyte. Besides these possible types, there are other cells, such as that in Plate II, figure 12, c, which appears similar to the first type mentioned above, but is smaller. The latter cells are all arbitrarily classed together with the first as a single type in the illustrative differential cell counts given in table 4. It should be emphasized that these differential cell counts are illustrative only and are based upon the tentative classification just given; in the last column of the table are all cells that can not be classed in the first three columns.

This table shows the feasibility of making differential blood cell counts from smears of uncoagulated blood. It also shows the possibility of most of the blood cells of *P. orientalis* consisting of one or two types.

#### DISCUSSION

It seems evident from the above observations and experiments that, in *P. orientalis*, blood coagulation is a process involving primarily changes in form and physico-chemical properties of the blood cells. During coagulation the latter round up, become more refractive, develop long and short pseudopodia, become "sticky", agglutinate and apparently disintegrate; under condition of mechanical agitation the cell forms become distorted and show a tendency toward formation of long bands of cell material. It is also evident, at least under the experimental conditions of the above observations and for periods up to several hours, that the plasma does not form microscopically visible fibers, that it undergoes relatively slight, if any, changes in viscosity when the factor of evaporation is eliminated and

TABLE 4. *Cell counts made from stained smears of uncoagulated cockroach blood*

Slide No.	Tentative cell types (See tentative classification given in text)			
	(1)	(2)	(3)	Other cells
102	72	2	3	23
103	80	2	16	2
104	88	1	5	6
106	85	7	8	0
107	91	1	2	6
108	79	18	3	0
110	96	0	4	0
111	94	0	4	2
112A	90	10	0	0
112B	98	2	0	0
112C	93	6	0	1
112D	91	9	0	0
112E	92	8	0	0
112F	93	6	0	1
160A	97	2	0	1
160B	97	2	1	0
160C	94	6	0	0
160D	97	3	0	0
Average	90	5	3	2

that there may appear in the plasma or serum of the clot a granular precipitate in active Brownian movement.

This description of cockroach blood coagulation as essentially a cell phenomenon is in close agreement with Loeb's (15) description of clotting in *Limulus* blood; in both cases the fibrous clot is of cellular origin and possesses the properties of stickyness, elasticity and, probably, contractility. On the basis largely of these properties, Loeb likens the cell coagulum of *Limulus* blood to the fibrin coagulum of mammalian blood. The facts (a) that the cockroach blood clot is primarily a cell coagulum and (b) that several authors have reported the detection of fibrin or a fibrin-like substance in cockroach and other insect bloods (Haber, Muttkowski, Landois, Cuenot) are not necessarily inconsistent, provided the cell coagulum possesses fibrin-like properties or contain fibrin as a constituent. On the other hand, it seems not improbable that some of the reported fibrin fibers may be in reality fibers of cell coagulum (Landois, Haber); figure 9 of Muttkowski's (19) paper could easily be of a cellular coagulum such as observed in the present work on *P. orientalis* blood. Landois' (14) description of fibrin could easily apply to a cell coagulum. Although it is not improbable that the blood of many insects may form coagula of fibrin fibers of plasma origin, the possibility of confusing thread pseudopodia and elongated bands of cell material with fibrin fibers should be emphasized.

It is of interest that the cell coagulum can be removed from a drop of blood with the point of a needle; this results in a great reduction in the number of cells in the drop. Loss of blood cells through coagulation may result in a very serious discrepancy in the true and experimental values



of total blood cell counts and may account for the very low estimates of total cell content of insect blood reported in the literature generally; for example, Haber (7) reports approximately two to three thousand cells per "droplet" of blood. On the other hand, there exists also the possibility of a slight cell coagulum remaining undetected. Such an occurrence may well explain conflicting reports in the literature; the blood of *Hydrophilus*, for example, has been reported to coagulate (19) and not to coagulate (1).

It has not been practicable in the present work to make satisfactory measurements of coagulation time, due to the difficulty of recognizing a suitable end-point; not only is the formation of the cell coagulum a gradual process, but it may occur somewhat irregularly over a large microscopic field. Comparative estimates of coagulation time in insects have been reported by Poulton and Griffiths, as noted above.

It has been shown that coagulation can be partially or completely inhibited by proper application of heat and that a sufficiently high temperature (70° C.) applied to the insect's body results in the appearance of a granular precipitate in the blood plasma. It has also been found that *P. orientalis* blood, taken from an unheated animal into a capillary glass tube, just shows a white precipitate at 64.5° C. The blood eventually solidifies so that it can not be forced from the tube. Similar observations were made by Poulton (21) on the blood of *S. ligustri* enclosed in a glass tube and immersed in a water bath; the blood became "slightly dim" at 132° F. (50.5° C.), "distinctly cloudy" at 141° F. (60.1° C.) and "quite coagulated" at 180° F. (80.3° C.). This author also reports the appearance of a plasma or serum precipitate upon dilution of the blood with distilled water or with alcohol. Barratt and Arnold (1) also noted a plasma precipitate in insect blood diluted with distilled water. In the case of *P. orientalis* blood, it is of interest that a plasma precipitate first appears at about 64.5° whether the blood be withdrawn and heated in a glass tube or heated while intact inside the animal's body; this would seem to indicate that heat coagulation of the plasma constituents is affected only slightly, if at all, by the blood coagulation process. Further work is necessary in order to answer the question whether the precipitate resulting from dilution of blood with distilled water, the precipitate resulting from heating the animal's body and the precipitate that occurs during the normal coagulation process are identical.

Blood smears have not been of general use in connection with insects, due presumably to the uneven distribution and obvious deformation of the blood cells; these effects are undoubtedly caused by the tendency of the blood to form a cell coagulum. Muttkowski (18) reports the use of blood smears "very unsatisfactory," but attributes the "clumping" and "undue distortion" of the cells to the viscosity of insect blood. In the experiments reported here, blood viscosity seemed to be a slightly interfering factor, tending to produce uneven cell distribution, only in those cases where the drop of blood was permitted to remain on the cut antenna for a period of one minute (a longer period was not employed) before smearing; in this case the drop of blood seemed to *dry* at its surface forming a gel-like but apparently non-fibrous membrane. As a result of the inadequacy of insect blood smears, the literature contains little information as to differential cell counts in insects. Counts are recorded by Metalnikov and Chorine (17) and other investigators of immunity in insects; usually, however, no

mention is made of cell distribution. Paillot's method (see above) of preventing coagulation should be useful in this respect, as well as the heat method described above; unfortunately, there has been no opportunity to apply Paillot's method during the present work.

This conception of cockroach blood coagulation as essentially a cell phenomenon is based upon observations of the animal's blood made under experimental conditions. The possibility that a true plasma coagulation may occur in the animal's body at a site of injury is not excluded, but is rendered less probable by the apparent lack of such plasma coagulation in blood observed *in vitro*. The above observations do not answer the question, to what extent does the blood plasma contribute to the formation of the cell coagulum? Although it is not impossible that constituents of the plasma may contribute toward the formation of the cell coagulum, these observations indicate that there is not in the blood of *P. orientalis* a definite secondary plasma coagulation such as has been described by Loeb as occurring in the blood of the lobster, *Homarus*, observed under similar experimental conditions. It seems probable that the following conditions might obtain during natural "hemotaxis" of cockroach blood occurring *in vivo*: blood cells stick to the injured tissue surfaces and to each other, forming a coagulum such as has been described; other cells, carried by the current of outflowing plasma, adhere to the mesh-work of the coagulum, until a layer of cells of appreciable thickness is formed over the injured region. The plasma flows less freely through the thickened meshwork of cells and so tends to *dry* at the exposed surface, forming a dried gelatinous membrane<sup>4</sup>; such a plasma change would probably correspond to the plasma "gelation" described by Muttkowski (19). The outflow of blood having stopped, new tissue may possibly be regenerated due to either (a) the cells of the coagulum, should the latter constitute a true "plasmodium," as conceived of by Geddes, or (b) new cells arriving at and adhering to the coagulum. This conception, for which no originality is claimed, is suggested here merely as a working hypothesis applicable to *P. orientalis*.

#### CONCLUSIONS

(1) Coagulation in the blood of the insect, *Periplaneta orientalis* (Linn.), consists essentially in the formation of a cellular coagulum by the blood cells.

(2) During the coagulation process many of the blood cells undergo the following changes: they lose their original fusiform or discoid shapes, round up, become more refractive, form thread pseudopodia, agglutinate into clumps of cells, spread out and seeming disintegrate (involving an apparent increase in viscosity of the protoplasmic material).

(3) The plasma (or serum) undergoes relatively little change that can be detected by microscopic observation; it may contain a granular pre-

---

<sup>4</sup>Evaporation at the exposed surface (a) of the drop of blood illustrated in figure 1, A, results in the formation of a gel-like membrane; the latter appears to be non-fibrous.

precipitate in active Brownian movement, but exhibits no fiber formation and seems to undergo little or no change in viscosity.

(4) The coagulation of *P. orientalis* blood appears to be of the same type as that of *Limulus* blood, described by Loeb, and conforms to the first of Tait's three types of arthropod blood coagulation given above.

(5) The coagulation of *P. orientalis* blood can be partially or completely inhibited by heating the animal to a sufficiently high temperature for a sufficient length of time; in these experiments the best results were obtained by heating the animal at 60° C. for ten minutes.

(6) Blood smears made from animals heated as described show a random distribution of blood cells. The cells are not distorted and apparently retain forms approximating the normal. Blood smears from unheated animals have unevenly distributed and distorted cells.

(7) It is possible to obtain differential cell counts from the randomly distributed blood smears.

(8) The following series of changes result from increasing the body temperature of *P. orientalis*: (1) an apparent slowing down of the coagulation process; (2) partial inhibition of coagulation; (3) complete inhibition of coagulation; (4) inhibition of coagulation and the appearance in the plasma of a granular precipitate; and (5) solidification of the blood in the animal's body.

#### REFERENCES

1. BARRATT, J. O., AND GEORGE ARNOLD  
1910. A study of the blood of certain coleoptera: *Dytiscus marginalis* and *Hydrophilus piceus*. *Quart. J. Micr. Sci.*, **56**:149.
2. BISHOP, G. A., A. P. BRIGGS AND E. RONZONI  
1925. Body fluids of honey bee larva. II. Chemical constituents of the blood, and their osmotic effects. *J. Biol. Chem.*, **66**:77.
3. CUENOT, L.  
1891. Le sang et les glandes lymphatiques dans la serie animale. *Arch de Zool. exp. et gen.*, **9**:365.
4. GEDDES, P.  
1880. On the coalescence of amoeboid cells into plasmodia, and on the so-called coagulation of invertebrate fluids. *Proc. Roy. Soc. London*, **30**:252.
5. GEYER, KURT  
1913. Untersuchungen über die chemische Zusammensetzung der Insektenhämolymphe und ihre Bedeutung für die geschlechtliche Differenzierung. *Ztschr. f. wiss. Zool.*, **105**:350.
6. GRIFFITHS, A. B.  
1891-2. On the blood of the invertebrata. *Proc. Roy. Soc. Edinburgh*, **19**:116.
7. HABER, VERNON R.  
1926. The blood of insects, with special reference to that of the common household german or croton cockroach, *Blattella germanica* Linn. *Bull. Brook. Ent. Soc.*, **21**:116.



8. HALLIBURTON, W. D.  
1885. On the blood of decapod crustacea. *J. Physiol.*, 6:300.
9. HARDY, W. B.  
1892. The blood corpuscles of the crustacea, together with a suggestion as to the origin of the crustacean fibrin-ferment. *J. Physiol.*, 13:165.
10. HAYCRAFT, J. B., AND E. W. CARLIER  
1888. On invertebrate blood removed from the vessels and entirely surrounded by oil. *Proc. Roy. Soc. Edinburgh*, 15:423.
11. HOBSON, A. D.  
1928. The effects of electrolytes on the muscle of the foregut of *Dystiscus marginalis*; with special reference to the action of potassium. *Br. J. Exp. Biol.*, 5:385.
12. HOLLANDE, A. CH.  
1907. Contribution a l' étude du sang des coléoptères. *Arch. de Zool. exp. et gen.*, 2:271.
13. \_\_\_\_\_  
1911. Étude histologique comparée du sang des insectes a hémorrhée et des insectes sans hémorrhée. *Arch. de Zool. exp. et gen.*, 6:283.
14. LANDOIS, H.  
1864. Beobachtungen über das Blut der Insekten. *Ztschr. f. wiss. Zool.*, 14:55.
15. LOEB, LEO  
1904. Über die Koagulation des Blutes einiger Arthropoden. *Beiträge zur Chem. Physiol. und Path.*, 5:191.
16. \_\_\_\_\_  
1927. Amoeboid movement and agglutination in amoebocytes of *Limulus* and the relation of these processes to tissue formation and thrombosis. *Protoplasma*, 2:512.
17. METALNIKOV AND CHORINE  
1929. On the natural and acquired immunity of *Pyrausta nubilalis* (corn borer). *International Cornborer Investigations, Scientific Reports*, 2:22.
18. MUTTKOWSKI, R. A.  
1924. Studies on the blood of insects II. The structural elements of the blood. *Bull. Brook. Ent. Soc.*, 19:4.
19. \_\_\_\_\_  
1924. Studies on the blood of insects III. The coagulation and clotting of insect blood. *Bull. Brook. Ent. Soc.*, 19:128.
20. PAILLOT, A.  
1923. Sur une technique nouvelle permettant l'étude vitale due sang des insects. *C. R. Soc. Biol.*, 88:1046.
21. POULTON, E. B.  
1885. The essential nature of the coloring of phytophagus larvae (and their pupa); with an account of some experiments upon the relation between the color of such larvae and that of their food plants. *Proc. Roy. Soc. London*, 38:269.
22. TAIT, JOHN  
1910-13. Types of crustacean blood coagulation. *J. Mar. Biol. Assoc.*, 9:191.
23. \_\_\_\_\_  
1918-20. Capillary phenomena observed in blood cells: thigmocytes, phagocytosis, amoeboid movement, differential adhesiveness of corpuscles, emigration of leucocytes. *Quart. J. Exp. Physiol.*, 12:1.



24. \_\_\_\_\_ AND J. DONALD GUNN  
1918-20. The blood of *Astacus fluviatilis*: a study of crustacean blood, with special reference to coagulation and phagocytosis. Quart. J. Exp. Physiol., 12:35.
25. YEAGER, J. FRANKLIN, AND OSCAR E. TAUBER  
1932. The determination of total blood volume in the cockroach, *P. fuliginosa*, with special reference to method. (In press.) Ann. Ent. Soc. Amer.

## PLATE I

The photographs of this plate were taken successively of the same microscopic field in a fresh preparation of the blood of *P. orientalis* in Nujol on a glass slide.

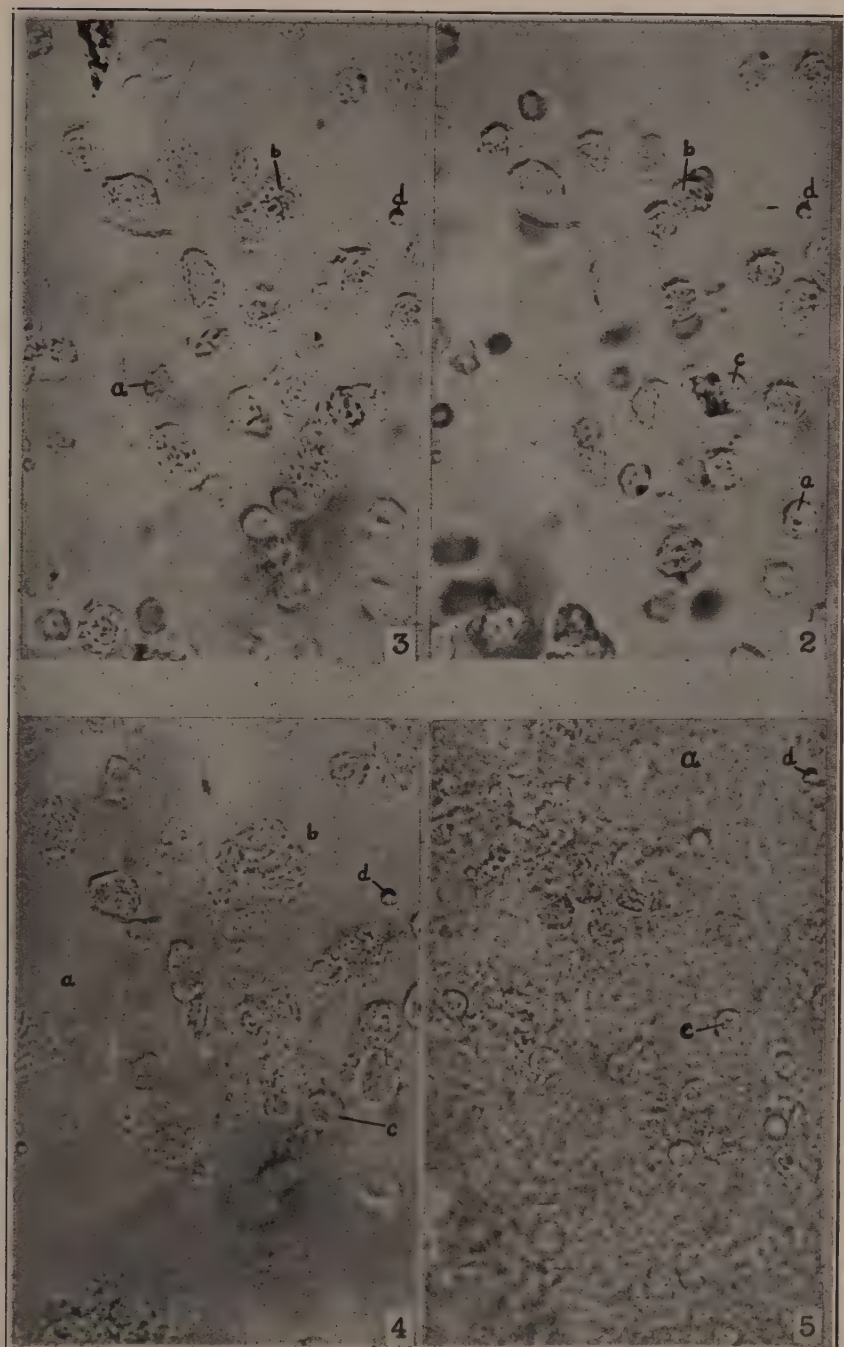
Fig. 2. Appearance of blood cells in blood plasma immediately after the drop of blood was immersed in the oil. The cells have lost their spindle shapes, have rounded up and have become more refractive; some are developing thread pseudopodia and some are beginning to agglutinate. The cells are still settling down to the surface of the slide.

Fig. 3. The same field a few minutes later. The cell changes begun in Fig. 2 are continuing. Many of the cells are agglutinated and are beginning to spread upon the surface of the slide. Note thread pseudopodia.

Fig. 4. The same field about one hour later. The agglutinated cells appear more spread out and more disintegrated, the clumps of cells beginning to assume a granular appearance.

Fig. 5. The same field about fourteen hours later. The clumps of cells are rendered less conspicuous by the large amount of granular precipitate that has appeared in the plasma; the particles of the precipitate are in active Brownian movement. The cell masses contain structures that may be cells or nuclei.

## PLATE I



## PLATE II

Fig. 6. Photograph of a fresh preparation of *P. orientalis* blood from an animal heated at 60° C. for ten minutes by immersion in water. The cells exhibit no signs of coagulation changes and seem to retain natural forms. Many contain large refractive granules, not very well shown in the figure.

Fig. 7. Photograph of fresh preparation of *P. orientalis* blood from an animal heated at 45° C. for ten minutes. The cells have rounded up, have become more refractive and have agglutinated; they do not show signs of disintegration or give other evidence of coagulation.

Fig. 8. Photograph of the edge of cellular coagulum in blood of unheated *P. orientalis*. Note the granular appearance of the coagulum (at *f*), the tendency of the cells to form broad bands of fibrous material (at *d*) and the granular precipitate in the plasma (at *e*); the latter is in active Brownian movement.

Fig. 9. Photograph of fresh preparation of *P. orientalis* blood from an animal heated at 60° C. for ten minutes by immersion in water. Note the fusiform appearance of the cells at *a* suspended in the fluid plasma; upon settling to rest upon the surface of the slide these cells appear like the cell seen at *b*.

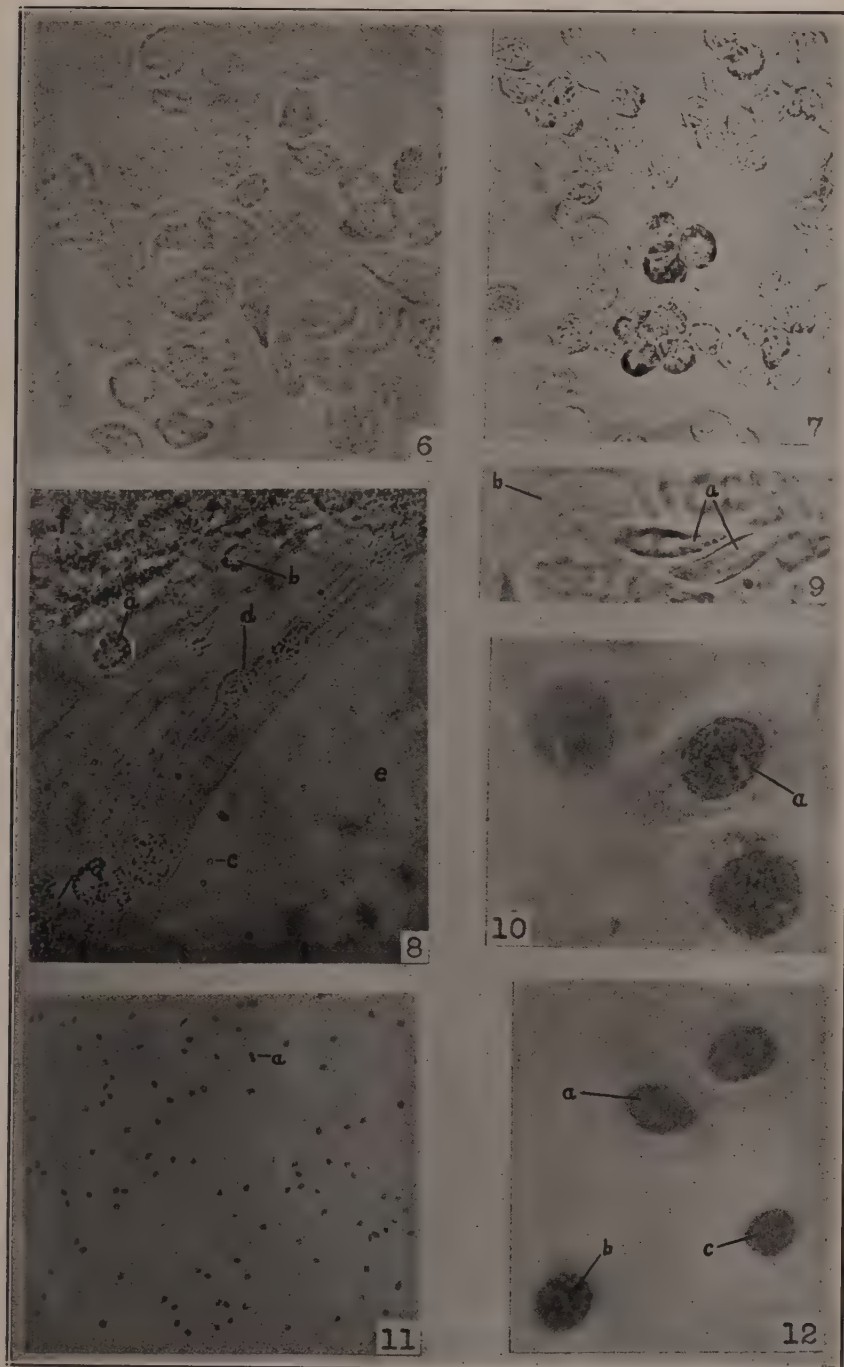
Fig. 10. Photograph of blood cells of *P. orientalis* made from a partially coagulated smear stained with Wright's blood stain. The cell at *a* is least disintegrated, while that above it is the most disintegrated of the three; note the more diffuse and more faintly stained appearance of the latter.

Fig. 11. Photograph of *P. orientalis* blood smear stained with Wright's blood stain; the blood was taken from an animal heated at 60° C. for ten minutes. Note the even distribution of blood cells and absence of cell disintegration. Note the dividing cell at *a*. Several circular spots in the plasma are due to fatty droplets in the blood.

Fig. 12. Photograph from smear like that shown in Fig. 11. Cells *a* and *c* appear similar except for size; they have basophilic nuclei and faintly staining, usually non-granular cytoplasm; the latter often contain small vacuoles that may represent fatty droplets. Cell *b* appears the same as cell *b* in Fig. 15, Pl. III.



PLATE II



## PLATE III

Fig. 13. Photograph of portion of a blood smear of *P. orientalis* blood taken from unheated animal and stained with Wright's blood stain. Coagulation is made evident by clumping, rounding up and intense basophilic staining of many blood cells. Note many eosinophilic cells, as cell *a* (the same cell as *b* in Fig. 15).

Fig. 14. Photograph from smear of unheated *P. orientalis* blood stained with Delafield's hematoxylin and eosin; note the fibrous and cellular nature of the coagulum.

Fig. 15. Photograph of portion of field shown in Fig. 13 (cell *a*). Note agglutinated cells at *a* and the cells at *b* showing an eosinophilic nucleus.

Fig. 16. Photograph from smear of unheated *P. orientalis* blood stained with Delafield's hematoxylin and eosin; note the fibrous and cellular nature of the coagulum.

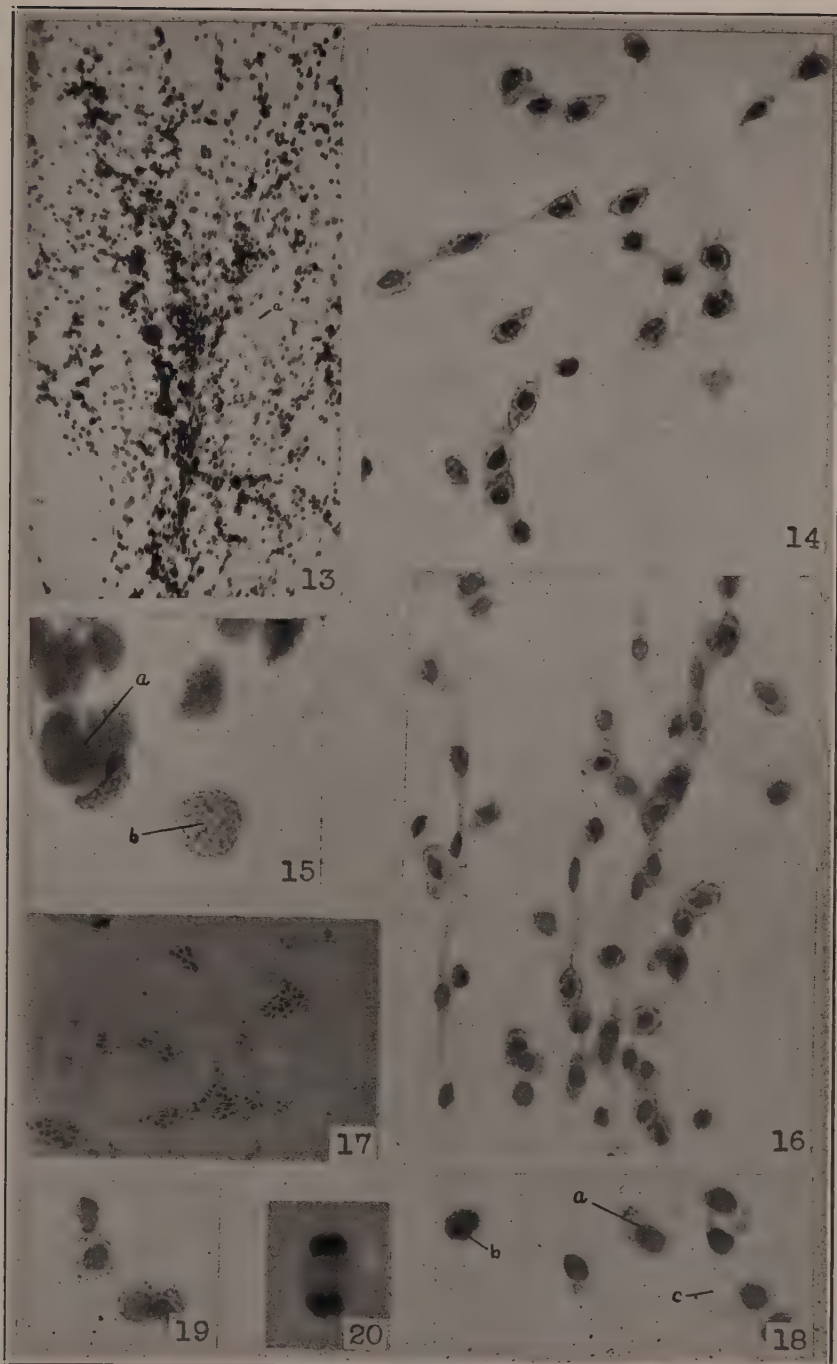
Fig. 17. Photograph of partially coagulated blood smear of *P. orientalis* blood. Note less intense coagulation changes as compared with the smear shown in Fig. 13.

Fig. 18. Photograph from smear of *P. orientalis* blood from animal heated at 60° C. for ten minutes; note different apparent cell types. Cell *a* similar to cells *a* and *c* of Fig. 12. Cell *b* appears similar to *b* in Fig. 12 and to *b* in Fig. 15.

Fig. 19. Photographs of cells in partially coagulated blood smear (*P. orientalis*) stained with Wright's blood stain. Note the eosinophilic granules in the peripheral cytoplasm. The cells appear similar to cell *a* in Fig. 18 and cell *a* in Fig. 10.

Fig. 20. Photograph of dividing cell in the smear shown as Fig. 11. The division seems to be mitotic.

PLATE III







# PRELIMINARY EXPERIMENTS WITH APHIDS AS VECTORS OF YELLOW DWARF<sup>1</sup>

C. J. DRAKE, H. D. TATE AND H. M. HARRIS

*From the Department of Zoology and Entomology, Iowa State College*

Accepted for publication April 20, 1932

Yellow dwarf is a comparatively new communicable disease of the cultivated onion and the literature pertaining to it is rather meager. Recently the writers (1) demonstrated the ability of certain species of plant lice to serve as agents for conveying this causal agent from diseased to healthy plants. In further work many significant facts concerning the transmission, development and behavior of the disease under greenhouse conditions have been determined.

Since the natural spread of yellow dwarf apparently depends solely upon its insect vectors, the rôle played by aphids in epidemics needs further perscrutation. Although many attempts to use the bulb mite (*Rhizoglyphus hyacinthi* Boisdual), the greenhouse whitefly (*Trialeurodes vaporariorum* Westwood), the red spider (*Tetranychus telarius* Linn.), the onion thrips (*Thrips tabaci* Lind.), springtails (Collembola), leaf bugs (Miridae), leaf hoppers (Cicadellidae), onion maggots (Anthomyiidae and Ortalidae), and numerous other insects as vectors of the disease have been made, the experiments have given negative results, except in a very few cases with the six-spotted leaf-hopper (*Cicadula sexnotata* Fall) and in one instance with a mealy bug (*Phenacoccus* sp.) found on greenhouse plants. On the other hand, many careful experiments in the greenhouse with the bean-aphid (*Aphis rumicis* Linn.)<sup>2</sup>, apple grain aphid (*Rhopalosiphum prunifoliae* Fitch), green peach aphid (*Myzus persicae* Sulzer), melon aphid (*Aphis gossypii* Glover), potato aphid (*Macrosiphum gei* Koch) and the corn leaf aphid (*Aphis maidis* Fitch) have positively shown that these species are conveyors of the yellow dwarf disease.

The present paper deals only with those experiments in which insects showed repeated ability to serve as vectors of yellow dwarf. In a subsequent article the experiments with insects which showed inability to serve as vectors of the malady will be discussed. Contrary to a statement in the recent literature the vectors of yellow dwarf are not confined to a local area in the state, but are widely disseminated in the United States and Canada. Some occur even on other continents. In the greenhouse studies the first transmissions of yellow dwarf were secured in December, 1930, by the corn leaf and the apple grain aphids.

A cursory examination of the publications relating to virus diseases of plants is sufficient to show the importance of members of the family Aphididae as active agents in the transmission of these diseases. Because of the unstable and at times almost insouciant feeding habits of the volant migrants, certain species of aphids are efficient carriers of the viruses of

<sup>1</sup>Journal Paper No. B53 of the Iowa Agricultural Experiment Station, Ames, Iowa.

<sup>2</sup>Aphididae determined by Dr. F. C. Hottes.

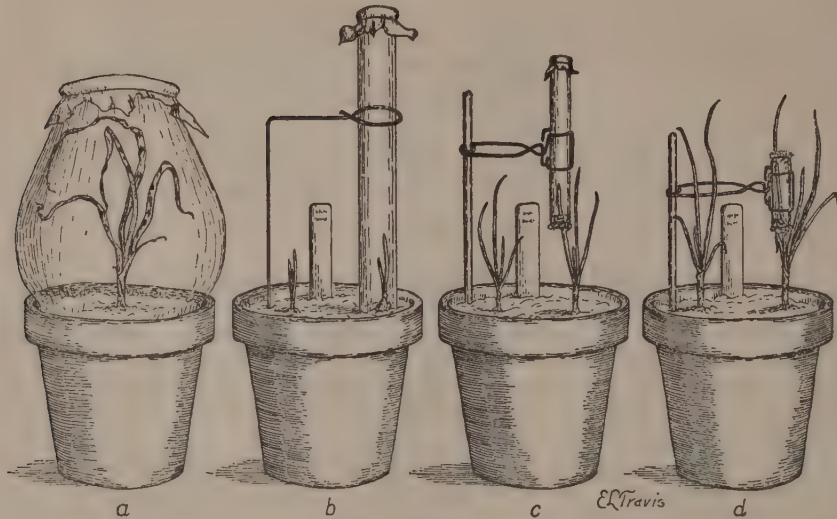


Fig. 1. Types of cages used for confining aphids on growing onions: *a*, aphids feeding upon diseased plant; *b*, *c* and *d*, cages adapted for confining viruliferous aphids upon entire or part of plants (control plants not caged).

many plant maladies. The behavior and migration of these insects are governed largely by their host specificities, periodic or seasonal host-restrictions, numerical abundance, high capacity for reproduction, and the availability of food plants. Some species are monophagous, whereas other forms are oligophagous or polyphagous and feed with various degrees of success upon many different kinds of plants during the growing season. Strangely enough, the secondary or summer hosts of polyphagous aphids serve only for parthenogenetic reproduction, for in the fall at the approach of cold weather, these species, like tourists returning from vacations, go back to the primary hosts. At this time or just before, the sexuparae appear and soon afterwards the sexuales. The oviparous females on the primary host then lay the eggs which serve to carry the species through the inclement season. Under field conditions in Iowa, most aphids spend the winter in the egg stage on branches of the primary host, which in the majority of cases is either a tree or shrub. In the experiments herein reported no work was done with the fundatrices, spuriae apterae, sexuales, or spring and fall migrants, the greenhouse studies being confined entirely to the agamic viviparous forms found on the summer host.

With favorable food plants and optimum conditions of temperature and relative humidity during the spring and summer, plant lice multiply very rapidly and often thickly populate and overrun their hosts. This overcrowding, together with the age and varying degree of succulency of food plants, tends to accelerate migration. In all species, however, there is in the winged individuals an inherent urge to wander so that under field conditions migration, which can neither be started, stopped, nor in any way greatly modified, occurs almost incessantly. Many graminivorous, herbivorous, and polyphagous species wander about in the spring, summer

and fall in search of new and less densely populated host plants, and thus tend to fill up any habitable gaps occurring in the environment.

In order to determine the distribution of the causal agent of yellow dwarf in the growing onion, aphids were confined on very limited sections (roots, bulbs, stems and various parts of young and old leaves such as the tip, central portion and base) of diseased sets and mother bulbs for a period of ten to twenty hours, after which they were transferred to healthy plants. In due time these showed unmistakable symptoms of the disease, thus proving that the infective principle resides in all these vegetative parts. Even wilting leaves, detached from diseased plants, served effectively as sources of inoculum. Parallel experiments, on the other hand, proved that healthy plants may be infected through the feeding of viruliferous aphids on the roots, bulb or stem, as well as on various parts of young and older leaves. Under certain field and greenhouse conditions the symptoms of yellow dwarf may be completely masked and not visible until after the bulbs have undergone a rest period and subsequently have been re-grown. In numerous tests it was found that masked plants, both young and old, are capable of infecting plant lice, which, when so contaminated, can convey the disease to healthy onions. In this connection it should be noted that those leaves on diseased plants which show no external evidence of the disease are also capable of serving as sources of inoculum for plant lice. Viruliferous seedlings, sets, mother bulbs and commercial onions in which the symptoms are masked thus may serve not only as a means of carrying the disease from season to season, but also as a source of infection for in-

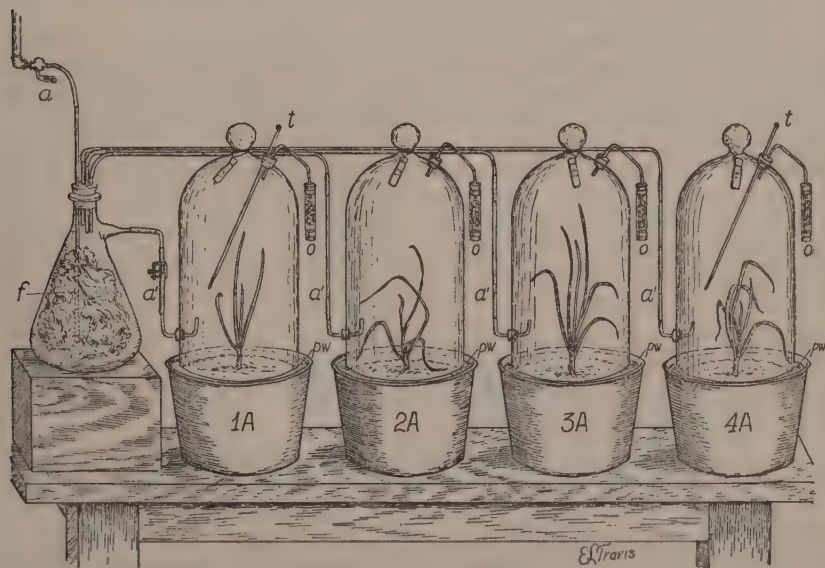


Fig. 2. Insect-proof cages: 1A and 3A, control plants; 2A and 4A, plants exposed to feeding of virus-bearing aphids; *a*, compressed air-line; *f*, cellulose cotton filter for removing oil from compressed air; *a'*, line for delivering air to cages; *o*, outlet of cages, showing cylinder containing glass-wool to keep out minute insects; *t*, thermometer.



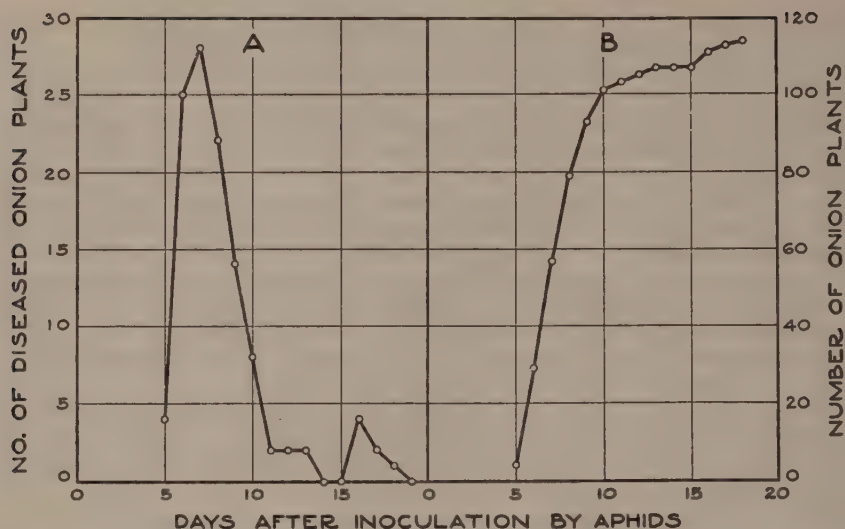


Fig. 3. Inoculation experiments in 120 set onions: A, showing daily initial expressions of yellow dwarf in 114 plants; B, total daily expressions of the disease in 114 out of 120 plants (6 plants failed to show symptom).

sect vectors during the first season as well as after the bulbs have been regrown the second year.

During their migrations and fortuitous wanderings in the spring, summer and fall, many aphids, especially markedly polyphagous species, by chance or necessity rest upon and imbibe the juices of plants which can function neither as primary nor secondary hosts. Because the aphids are not able to breed or even live for any period of time upon them these ephemeral host plants serve purely as temporary sources of nourishment for the shifting migrants. The onion thus may be termed a transient host, merely serving to help sustain the wandering lice during migration. In the case of yellow dwarf the migrating aphid may convey the virus from diseased to healthy onions, while the plant serves solely as a temporary host for the transients. When confined on the onion plants, nymphs and alate and apterous plant lice perish within a period of from three to six days; in fact, young nymphs, either born upon or transferred to the leaves of onion, die before attaining the adult stage, and mature viviparous females soon stop reproducing and perish. Some species, however, imbibe the juices much more freely than others.

The aphids used in the greenhouse experiments consisted of summer generations composed largely of apterous viviparae. Since the cultivated host plants were not susceptible to the disease, and since the aphids were colonized in separate rooms, there was little danger of aphids or onions becoming accidentally infected with the virus prior to the transmission experiments. From time to time non-infected aphids were caged on healthy onions as controls. Other plants not exposed to attacks of aphids were also used as controls. The total number of these control plants amounted to nearly twice that of the experimental ones, but in no instance did they



show any symptoms of yellow dwarf. In all of the experiments efforts were made to use young and vigorous aphids from small colonies feeding upon healthy hosts. At regular intervals new colonies were started so as to maintain a continuous supply of the insects. Yellow bottle-neck onion sets of a uniform size, grown at Clear Lake, Iowa, and free from yellow dwarf virus, were used in the experiments. In order to have a succession of young onions for experimentation some sets were planted every two or three days. After onions have been grown in a greenhouse for several weeks thrips become extremely abundant and make it imperative that a definite spray program be followed. The plants, therefore, were sprayed regularly at intervals of three days with nicotine sulphate or pyrethrum-soap solution to reduce the population of onion thrips. The experimental plants were caged only during the inoculating period. On the fourth day the cages were removed and both plant and cages were carefully sprayed to make certain that no plant lice escaped in the experimental room.

After healthy plants have been exposed to viruliferous aphids, the first visible expressions of yellow dwarf generally appear within a period of from five to twelve days. In a series of carefully planned experiments aphids were found to be very efficient conveyors of the virus of yellow dwarf. The results of some of these experiments are given in figures 3 and 4. Viruliferous aphids (Fig. 3) were confined on 120 plants and 114, or 95 per cent of these set onions, developed typical symptoms of the dis-

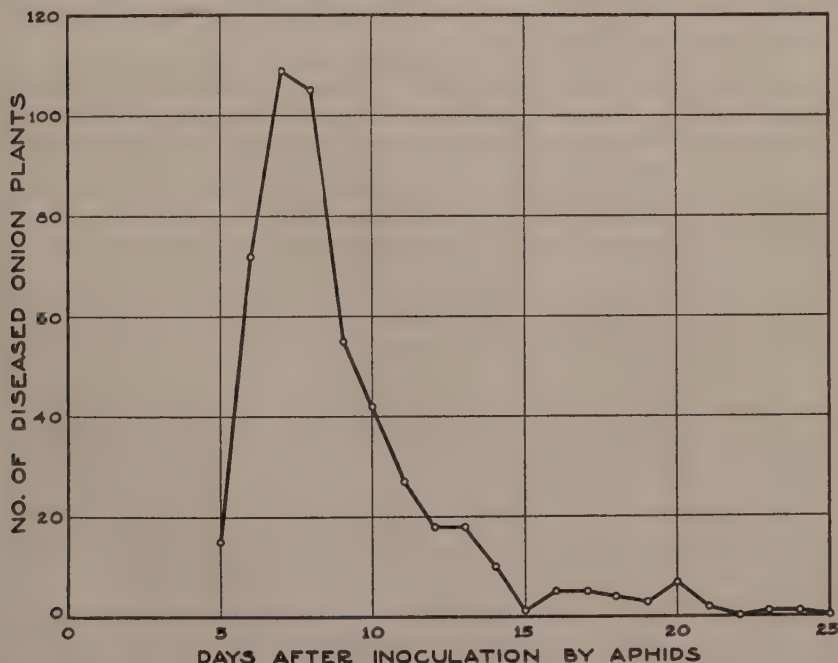


Fig. 4. Showing daily symptom expressions of yellow dwarf disease in 500 set onions inoculated by *Aphis rumicis*.

ease. The remaining six bulbs have not as yet been tested to see whether the disease was in masked form or whether the aphids failed to inoculate these plants. The length of time necessary for the appearance of the diagnostic characteristics of the disease in 500 transmissions to set onions by *Aphis rumicis* (Linn.) is depicted in the accompanying chart (Fig. 4). In this connection it is interesting to note that in slightly over 42 per cent (214) of the sets the first visible symptom expressions of the disease appeared on the seventh and eighth days after exposure to virus-bearing aphids. As was evidenced by a few stragglers, a small percentage of the plants had an arrested or prolonged development of the infection resulting in a temporary masking or restriction of the external manifestations of the disease.

In many tests using one, two, three, four and five infective aphids, respectively, it was found that one individual was capable of inoculating a plant, but the percentage of transmission under such conditions was much less than when a higher number was used. Aphids caged for thirty minutes on diseased plants and then transferred individually to healthy onions for a similar period of time successfully inoculated them, thus in a way demonstrating the extreme infectiousness of the disease, as well as proving conclusively that wandering plant lice may become carriers or, if already infective, inoculate plants by single feedings. In these preliminary tests using a few or an individual aphid as compared with those using a series of ten to fifteen there seemed to be no appreciable difference in the incubation period in the plant as evinced by the first appearance of unmistakable expressions of the disease.

It was very patent that some individuals of a given species do not feed as readily on the onion as others. In order to feel fairly certain that feeding actually occurred it was found of value in the majority of cases to use a series of from ten to fifteen aphids per plant in the inoculation tests. In some cases from 50 to 100 or more infective plant lice were caged on a healthy plant, but the visible expressions of the disease did not manifest themselves any sooner than in those plants where the usual number was used. In a long series of tests infective aphids were transferred from one disease-free plant to another at intervals of four hours for a period of several days. After a few transfers the aphids became non-infective. In these tests there was either a rapid decrease or a sudden stop in the number of infections after the second transfer.

The appearance and reactions of the onion plant after exposure to the feeding of virus-bearing plant lice were observed carefully and recorded each day. These daily observations were made between eight and ten o'clock by the same individual so as to give uniformity in the tests and, hence, comparable data. In most instances the aphids were infected by being caged on relatively new foliage of plants showing typical symptoms of yellow dwarf. In one instance a diseased onion, used as a source of inoculum over a period of a few weeks, served for infecting more than 10,000 aphids without exhausting the supply of virus; from this test the rôle a single diseased onion may play in epidemics of yellow dwarf is quite apparent. The plants were grown in a room kept at temperatures sufficiently low for the development of good onions, whereas the insects were reared in other rooms at higher temperatures favorable for their rapid multiplication. Everything considered, the months of February, March and April

were most suitable for these studies under greenhouse conditions. The greenhouse was lighted artificially (250 watt bulbs) on cloudy days and at night until eleven o'clock during the months of February and March.

The original culture of the bean aphid (*Aphis rumicis* Linn.) used in the long series of experiments was secured from Dr. F. L. Campbell, Bureau of Entomology, Washington, D. C. This race feeds on nasturtium and has been reared under greenhouse conditions for many years for toxicological and insecticidal tests by Richardson, Campbell, Shepard and others.

It is a well established fact that the virus of yellow dwarf overwinters in diseased sets, mother bulbs and commercial onions. Such bulbs, together with diseased scullions and culls thrown in refuse piles and dump heaps or left in the field, may serve as sources of inoculum for the vectors the following season. Because of the early development and appearance of typical symptoms in diseased bulbs regrown the subsequent spring, the relation of such bulbs to the spread of yellow dwarf is quite evident. Since no perennial wild host has as yet been found in the fields of Iowa, the diseased onion bulb appears to be the true reservoir of the overwintering virus.

Aphids are capable of conveying the infection to healthy onions almost immediately after having fed upon a diseased plant. Conversely, on the third or fourth day after being bitten by infective plant lice, the onion becomes a source of infection for aphids. After this short incubation of three to four days, diseased plants remain a constant source of inoculum as long as the aphids are able to extract the juices from them. Such diseased sets or bulbs, when regrown the following spring or left in the field after harvest to sprout and grow as volunteers, again become sources of infection for the vectors. On the other hand, aphids soon lose the virus unless constantly reinfected from diseased plants; after being caged on wild or cultivated hosts, or after a few transfers from healthy to healthy onions, they also become non-infective.

Viruliferous aphids were caged on set onions soon after the tips of the new leaves appeared. In many instances the first expressions of the disease showed on such sets while the bulbs were still in the three-leaf stage. Mature or nearly mature plants and slow-growing and unhealthy ones do not seem to be so easily infected as healthy and rapidly-growing young onions. More masking also occurs in the older plants than in newly started sets, mother bulbs and young seedlings. The degree of injury depends considerably upon the stage of growth and size of the bulb when the vectors inoculate its foliage. If infected soon after the new foliage appears, sets frequently fail to make any appreciable growth. The principal losses are due to the failure of diseased plants to manufacture and store sufficient food for the development of commercial onions. Since the actual sources of yellow dwarf infection at the beginning of each season are onion bulbs infected the previous year, and since the spread of the virus from diseased to healthy plants depends solely upon insect vectors, the control of the disease is largely a matter of preventing infection of the new crop from the overwintering virus and its subsequent wholesale dissemination by its insect vectors.

Spraying to prevent the spread of yellow dwarf by insect vectors is expensive and has extremely limited possibilities. Complete destruction of all crop remnants along with the use of disease-free sets produced in



non-infected areas constitutes the most practical remedial measure. Since the disease is not seed borne and since aphids are delicate flyers and also soon become non-viruliferous when removed from diseased plants, it is possible to produce disease-free sets within a few miles of infected fields. In the experiments for the control of onion thrips and maggots, many different combinations of spray materials have been used. Indexing of the sets produced in these experimental plots has disclosed that certain sprays had a noticeable effect in lessening the percentage of yellow dwarf infection. The foremost of these sprays was a combination of miscible oil (1-1.5 per cent) in bordeaux mixture (4-4-50) with pyrethrum soap (1-1000) or nicotine-sulfate (1-1000). Three to five applications of the sprays were made at weekly intervals. Those sprays which might have served to kill, or repel the aphids for several days, seemed to give better results than those containing more quickly-disappearing contact ingredients. In some of the check plots the infection with yellow dwarf was as great as 60 per cent, whereas in the plots sprayed with the above mentioned material infection was reduced to less than two per cent.

#### BIBLIOGRAPHY

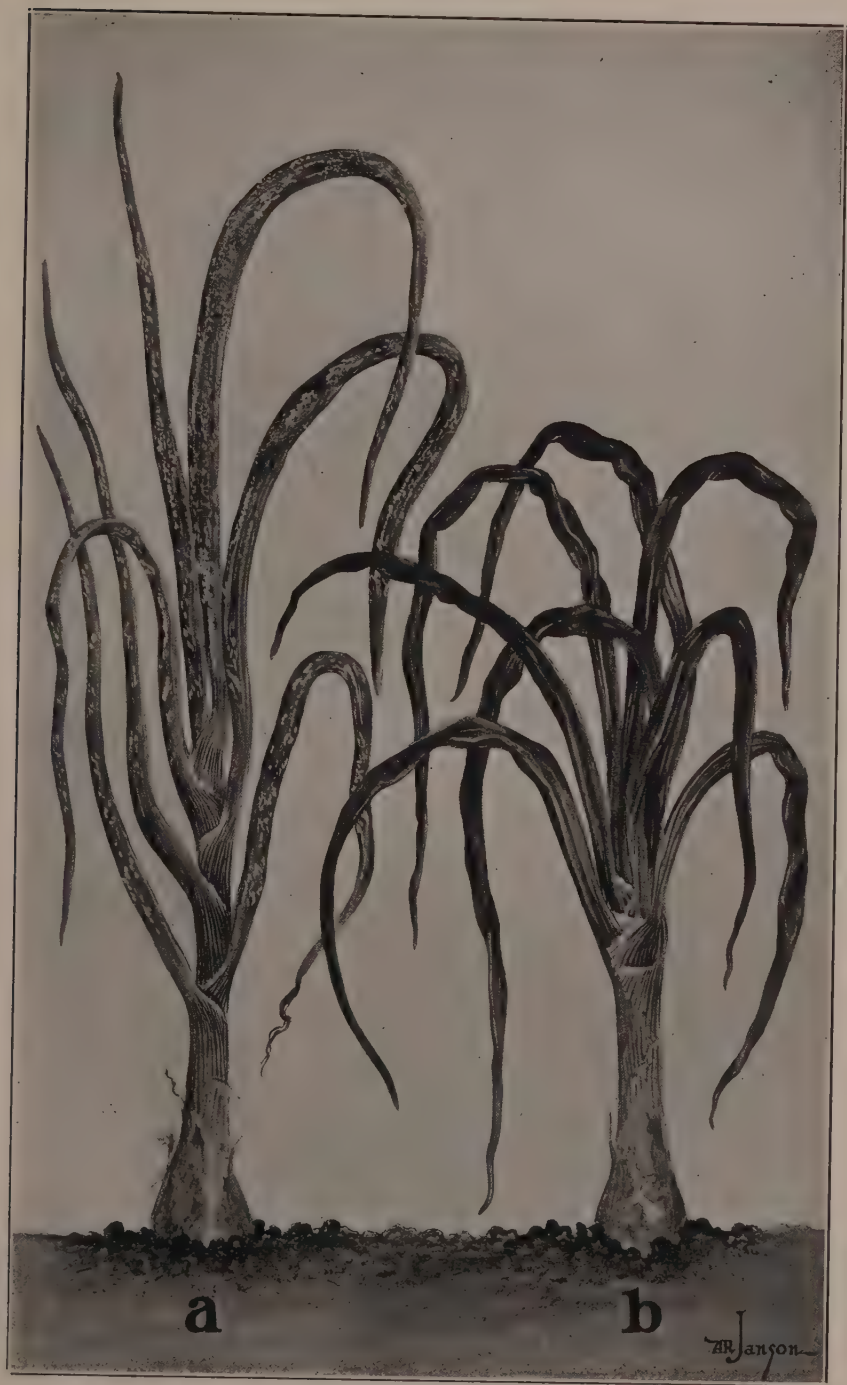
1. DRAKE, C. J., H. M. HARRIS AND H. D. TATE  
1932. Insects as vectors of yellow dwarf of onions. *Science*, 75:341-342.
2. HENDERSON, W. J.  
1932. Studies of the properties and host reaction of the onion to the yellow dwarf virus. (Abstract.) *Phytopath.*, 22:11.
3. MELHUS, I. E., C. S. REDDY, W. J. HENDERSON AND EDGAR VESTAL  
1929. A new virus disease epidemic on onions. *Phytopath.*, 19:73-77.
4. ————— AND W. J. HENDERSON  
1929. The yellow dwarf of onions. (Abstract.) *Phytopath.*, 19:86.
5. —————  
1932. Yellow dwarf and other onion diseases. Report on agricultural research for year ending June 30, 1931, Iowa Agr. Exp. Sta., p. 49.
6. PORTER, D. R., AND W. J. HENDERSON  
1929. Onion diseases. *Trans. Iowa Hort. Soc.*, 63:240.

#### PLATE I

Onion plants showing (a) typical feeding injury by the onion thrips and (b) yellow dwarf disease.



PLATE I





# FIRST SUPPLEMENTARY LIST OF PARASITIC FUNGI FROM IOWA

JOSEPH C. GILMAN

*From the Department of Botany, Iowa State College*

Accepted for publication May 19, 1932

Since the publication of the earlier list (29) of the parasitic fungi from the state, in 1929, further collections and publications have increased our list to such an extent as to make advisable a supplementary report. Thirty-five fungi unrecorded in the state have been collected and twenty-six hosts have been added as subject to parasitic invasion. With these are listed the additional hosts for parasites already reported and additional parasites on hosts previously found. These additions bring the total number of new associations of the host and parasite in the state to seventy-seven.

Few additions to the parasitic flora of Iowa have been recorded in the literature during the period under consideration. Wilson (51) has added the most names to the list with three parasites and three hosts. The other contributions have been made chiefly from collections by the staff of the Botany Department at Iowa State College, whose assistance, together with that of Dr. H. S. Conard and of Dr. G. W. Martin of the Lakeside Laboratory at Lake Okoboji, is gratefully acknowledged.

The hosts in this list all belong to families of plants already recorded (29). Of the fungi, members of the genera *Amerosporium*, *Aspergillus*, *Cladotrichum*, *Glomerularia*, *Phlyctaena*, *Rhinotrichum*, *Rhynchosporium* have not been previously reported for Iowa. To sum up, the total number of hosts in the state now comprises 1035 species, while the fungi number 995. The species not hitherto listed are marked with an asterisk. The figures in parenthesis after the fungus name refer to a citation in which the fungus is described, after the host name to a record of the fungus from Iowa.

1. *Albugo candida* (Pers.) Rouss. (51)  
On *Brassica juncea* L. Carroll: Pammel, 1928.
2. \**Amerosporium oeconomium* Ell. & Tracy (24)  
On *Vigna sinensis* Endl. Muscatine: Layton, 1928.
3. *Armillaria mellea* (Fr.) Quelet (33)  
On *Quercus rubra* L. Des Moines: R. H. Porter, 1929.
4. \**Aspergillus niger*. Van Tiegh. (48)  
On *Allium cepa* L. (cult.) Pleasant Valley: Henderson, 1929.
5. *Botrytis cinerea* Pers. (45)  
On *Ranunculus abortivus* L. Ames: Buchholtz, 1930.
6. \**Botrytis tulipae* (Lib.) Hopkins (32)  
On *Tulipa* sp. (cult.). Wapello: R. H. Porter, 1932.
7. *Cercospora granuliformis* Ell. & Holw. (26)  
On *Viola tricolor* var. *hortensis* D. C. Webster City: R. H. Porter, 1929.
8. \**Cercospora mirabilis* Tharp. (47)  
On *Mirabilis jalapa* L. Muscatine Co.: Layton, 1928.

9. *Cicinnobolus cesati* De B. (2)  
On *Erysiphe polygoni* D. C. on *Lycium halimifolium* Mill. Ames: Gilman, 1931.
10. *Contractia caricis* (Pers.) Magn. (11, p. 33)  
On *Carex grisea* Wah. Lee County: Fults, 1931.
11. \**Cladotrichum leersiae* Atk. (6)  
On *Leersia oryzoides* (L.) Sw. Arnold's Park: Gilman, 1931.
12. \**Claviceps nigricans* Tul. (5)  
On *Eleocharis palustris* (L.) R. & S. Clarion: Melhus, 1929.  
cf. Krieger, Fungi Saxonici No. 865.
13. *Colletotrichum graminicolum* (Ces.) Wils. (52)  
On *Poa pratensis* L. Ontario: Gilman, 1931.
14. \**Colletotrichum violarum* J. J. Davis (16)  
On *Viola sororia* Willd. Estherville: Gilman, 1931.
15. *Cordyceps* sp.  
On *Luperina stipata* Morr. Ames: Decker, 1930. (19)
16. \**Cordyceps calvulata* Schw. (38)  
On *Lecanium* sp. Ames: Burroughs, 1930.
17. *Cronartium ribicola* Fisch. de Waldh. (4, pp. 122 and 692)  
On *Ribes* sp. (cult. black currant). Centerpoint: Ness, 1929.
18. *Cylindrosporium apocyni* Ell. & Ev. (13)  
Syn. *Stagnospora apocyni* (Pk.) Davis.  
On *Apocynum cannabinum* L. Osage: McDonald, 1929.
19. *Cylindrosporium salicinum* (Pk.) Dearn. (17)  
On *Salix interior* Rowlee (*Salix longifolia* Auct.). Ames: Simonds. 1931.
20. *Discosia artocreas* (Fr.) Tode (20)  
On *Vitis vulpina* L. Ames: Sylvester, 1931.
21. *Dothichiza populea* Sacc. (31)  
On *Populus deltoides* Marshall (Carolina poplar). Ames: Gilman, 1930.
22. *Erysiphe cichoracearum* DC. (42)  
On *Citrullus vulgaris* L. (greenhouse). Ames: Weetman, 1932.  
On *Cucumis melo* L. Muscatine Co.: Layton, 1928.  
On *Zinnia elegans* Jacq. Blairstown: Pammel, 1929.
23. *Erysiphe graminis* DC. (42)  
On *Agropyron repens* (L.) Beauv. Ames: Hershey, 1930.
24. *Erysiphe polygoni* DC. (42)  
On *Lycium halimifolium* Mill. Ames: Gilman, 1931.
25. \**Glomerularia corni* PK. (37) L.  
On *Lonicera tatarica* L. var. *alba* Hort. Ames: Melhus, 1929.  
cf. Ellis N. Amer. Fungi 1230, Barth. Fungi Columb. 2325.
26. *Gymnosporangium globosum* Farl. (4, p. 204)  
On *Juniperus scopulorum* Sarg. Ames: Bliss, 1930.  
On *Pyrus communis* L. (Cult. pear). Kent: Bliss, 1930.
27. *Gymnosporangium juniperi-virginianae* Schw. (4, p. 209)  
On *Juniperus scopulorum* Sarg. Ames: Bliss, 1930.
28. \**Helminthosporium bromi* Diedicke (22)  
On *Bromus inermis* Leyss. Ames: Gilman, 1931.
29. *Helminthosporium stenacrum* Drechsler (22)  
On *Agrostis maritima* Lam. Shenandoah: R. H. Porter, 1931.



30. *Helminthosporium* sp.  
On *Bouteloua oligostachya* (Nutt.) Torr. *Lake Okoboji*: Gilman, 1931.
31. *Macrosporium cucumerinum* Ell. & Ev. (8)  
On *Cucumis melo* L. (cult. muskmelon). *Janesville*: Layton, 1931.
32. \**Macrosporium porri* Ell. (3)  
On *Allium cepa* L. *Homestead*: Henderson, 1929.
33. \**Marssonina sonchi* Dearn. & Bisby (18)  
On *Sonchus arvensis* L. *Mason City*: Vestal, 1930.
34. \**Melanconis juglandis* (Ell. & Ev.) Graves (30)  
On *Juglans nigra* L. *Ames*: Gilman, 1931.
35. \**Metarrhizium anisopliae* (Metsch.) Sorokin (38)  
On *Arcanellus* sp. *Ames*: Decker, 1930.
36. *Mosaic* (21)  
On *Nicotiana affinis* T. Moore. 1927
37. \**Peronospora claytoniae* Farl (27)  
On *Claytonia virginica* L. (51).
38. \**Peronospora dicentra* Sydow (28)  
On *Dicentra cucullaria* L. *Ames*: Layton, 1930. *Winterset*: Gilman, 1932.
39. \**Peronospora plantaginis* Underw. (49)  
On *Plantago aristata* Michx. (51)
40. *Phragmidium uredinis* Link (4, p. 186)  
Syn. *Kuehneola uredinis* (L. K.) Arth.  
On *Rubrus procumbens* Muhl. (51)
41. \**Phlyctaena linicola* Speg. (7)  
On *Linum usitatissimum* L. (cult. var. Bison). *Ames*: Reddy, 1931.
42. \**Phyllosticta catalpae* Ell. & Mart. (43)  
On *Catalpa bignonioides* Walt. *Conesville*: Layton, 1929.
43. \**Phyllosticta persicae* Sacc. (43)  
On *Prunus persica* L. *Conesville*: Layton, 1929.
44. *Phyllosticta phaseolina* Sacc. (43) (Benth) Wats.  
On *Strophostyles pauciflora*. *Conesville*: Melhus, 1930.
45. \**Phyllosticta podophylli* (M. A. Curt.) Wint.  
On *Podophyllum peltatum* L. *Ames*: Weetman, 1932.  
cf. Ellis N. Am. Fung. 1156.
46. \**Phyllosticta quercus* Sacc. and Speg. (43)  
On *Quercus prinoides* Willd. *Winterset*: Gilman, 1930.
47. *Podosphaera oxyacanthae* (DC.) DeBy. (42)  
On *Prunus virginiana* L. *Ames*: Ellis, 1930.
48. *Pseudoperonospora cubensis* (B. & C.) Rostow. (40)  
On *Cucurbita pepo* L. *Conesville*: Layton, 1928.
49. *Puccinia asterum* Kern (4, p. 362)  
On *Dulichium arundinaceum* (L.) Britton. *Winnebago Co.*: Pammel, 1908.
50. \**Puccinia emiliae* P. Henn. (4, p. 584)  
On *Calendula officinalis* L. *Ames*: Berberian, 1931.  
On *Dimorphotheca cuneata* (cult.). *Ames*: Berberian, 1931.
51. *Puccinia graminis* Pers. (4, p. 295)  
On *Cinna arundinacea* L. *Grinnell*: Conard, 1927.

52. *Puccinia menthae* Pers. (4, p. 405)  
On *Mentha gentilis* L. *Grinnell*: Conard, 1923.  
This was reported previously as "Mentha sp."  
On *Pycnanthemum flexuosum* (Walt.) BSP. (51)
- 52a. *Puccinia seymouriana* Arth. (4, p. 318)  
On *Cephalanthus occidentalis* L. *McGregor*: Hendershott, 1932.
53. *Puccinia sorghi* Schw. (4, p. 277)  
On *Euchlaena mexicana* Schrad. *Ames*: Gilman, 1931.
- 53a. \**Puccinia tubulosa* (Pat. and Gaill.) Arth. (4, p. 288)  
On *Solanum carolinense* L. *Conesville*: Layton, 1932.
54. *Pucciniastrum americanum* (Farl.) Arth. (4, p. 677)  
On *Rubus* sp. (cult. var. Latham red raspberry).
55. \**Rhinotrichum doliolum* Pound and Clements (10)  
On *Diderma floriforme* Pers. *Boone, Ledges*: Weetman, 1931.
56. \**Rhynchosporium alismatis* (Aud.) J. J. Davis (15)  
On *Alisma plantago-aquatica* L. *Lee Co.*: Fults, 1931.
57. *Sclerotinia bifrons* (Ell. & Ev.) Seaver and Shope (44)  
On *Populus tremuloides* Michx. *Allamakee Co.*: Miller, 1929.
58. *Sclerotinia libertiana* Fekl. (55)  
On *Aquilegia* sp. (cult.). *Ames*: Bode, 1930.
59. \**Sclerotinia smilacinae* Durand (23)  
On *Smilacina racemosa* (L.) Desf. *Ames*: Davis, 1930.
60. \**Septoria atriplicis* Desm. (16)  
On *Chenopodium album* L. *Milford*: Gilman, 1931.
61. *Septoria bataticola* Taub. (46)  
On *Ipomoea hederacea* Jacq. *Muscatine Co.*: Layton, 1928.
62. \**Septoria citrulli* Ell. & Ev. (24)  
On *Citrullus vulgaris* L. *Conesville*: Layton, 1928.
63. \**Septoria coreopsidis* J. J. Davis (14)  
On *Coreopsis tripteris* L. (50). *Oskaloosa*: G. W. Wilson, 1929.
63. \**Septoria dracocephali* Thüm. (41, v. 3, p. 540)  
On *Dracocephalum parviflorum* Nutt. *Spirit Lake*: R. H. Porter, 1931.
65. *Septoria helianthi* Ell. & Kell. (36)  
On *Helianthus annuus* L. *Conesville*: Layton, 1930.
66. \**Septoria mitellae* Ell. & Ev. (35)  
On *Mitella diphylla* L. *Iowa City*: Austin, 1930.
67. \**Septoria septentrionalis* H. W. Anderson (1)  
On *Ranunculus* sp. *Ames*: Kopf, 1932.
68. *Septoria virgaureae* Desm. (36)  
On *Solidago latifolia* L. *Milford*: Gilman, 1931.
69. *Sporodinia grandis* Link (35)  
On *Secotium agaricoides* (Czern.) Hollos. *Ames*: Gilman, 1929.
70. \**Sporotrichum globuliferum* Speg. (38)  
On *Arcanellus* sp. *Ames*: Doecker, 1930.
71. \**Stagonospora caricinella* Brun (12)  
On *Carex pennsylvanica* Lam. *Ames*: Gaskill, 1932.
72. \**Stagonospora paludosa* (Sacc. & Speg.) Sacc. (41, v. 3, p. 453)  
On *Carex Jamesii* Schw. *Ames*: Hershey, 1930.
73. *Taphrina coerulescens* (Mont. & Desm.) Tul. (50)  
On *Quercus macrocarpa* W. *Okoboji*: G. W. Martin, 1929.  
On *Quercus velutina* Lam. *Cresco*: Pammel, 1929.

74. *Tuberculina persicina* (Ditm.) Sacc. (41)  
On *Aecidium onobrychidis* Burrell on *Apios tuberosa* Moench. (51).
75. *Uncinula salicis* (DC.) Wint. (42)  
On *Salix nigra* Marsh. Ames: Nagel, 1931.
76. *Urocystis anemones* (Pers.) Schroet. (11, p. 55)  
On *Anemonella thalictroides* (L.) Spach. Ames: Hershey, 1930.
77. *Uromyces lespedezae-procumbentis* (Schw.) Curt. (4, p. 247)  
On *Lespedeza violacea* (L.) Pers. Eagle Rock: Pammel, 1920.  
On *Lespedeza virginica* (L.) Britt. Lacey-Keosauqua Park: Pammel, 1929.
78. \**Uromyces punctatus* Schroet. (4, p. 253)  
On *Astragalus canadensis* L. Ames: Gilman, 1929.
79. \**Ustilago lorentziana* Thuem (11, p. 9)  
On *Hordeum jubatum* L. (51). Swea City: Robt. Brown, 1930.
80. *Yellows* (38)  
On *Fragaria* sp. (cult. strawberry). Ames: R. H. Porter, 1929..

## HOST INDEX

- |   |  |
|---|--|
| * <i>Aecidium onobrychidis</i> Burrill        | <i>Cinna arundinacea</i> L.                  |
| <i>Tuberculina persicina</i>                  | <i>Puccinia graminis</i>                     |
| <i>Agropyron repens</i> (L.) Beauv.           | <i>Citrullus vulgaris</i> L.                 |
| <i>Erysiphe graminis</i>                      | <i>Erysiphe cichoracearum</i> DC.            |
| * <i>Agrostis maritima</i> Lam.               | <i>Septoria citrulli</i>                     |
| <i>Helminthosporium stenacrum</i>             | <i>Claytonia virginica</i> L.                |
| <i>Alisma plantago-aquatica</i> L.            | <i>Peronospora claytoniae</i>                |
| <i>Rhynchosporium alismatis</i>               | * <i>Coreopsis tripteris</i> L.              |
| <i>Allium cepa</i> L.                         | <i>Septoria coreopsidis</i>                  |
| <i>Aspergillus niger</i>                      | <i>Cucumis melo</i> L.                       |
| <i>Macrosporium porri</i>                     | <i>Erysiphe cichoracearum</i>                |
| <i>Anemonella thalictroides</i> (L.) Spach.   | <i>Macrosporium cucumerinum</i>              |
| <i>Urocystis anemones</i>                     | <i>Cucurbita pepo</i> L.                     |
| * <i>Apocynum cannabinum</i> L.               | <i>Pseudoperonospora cubensis</i>            |
| <i>Cylindrosporium apocyni</i>                | <i>Dicentra cucullaria</i> L.                |
| <i>Aquilegia</i> sp.                          | <i>Peronospora dicentrae</i>                 |
| <i>Sclerotinia libertiana</i>                 | * <i>Diderma floriforme</i> Pers.            |
| * <i>Arcanellus</i> sp.                       | <i>Rhinotrichum dolium</i>                   |
| <i>Metarrhizium anisopliae</i>                | * <i>Dimorphotheca cuneata</i> (cult.)       |
| <i>Sporotrichum globuliferum</i>              | <i>Puccinia emiliae</i>                      |
| <i>Astragalus canadensis</i> L.               | * <i>Dracocephalum parviflorum</i> Nutt.     |
| <i>Uromyces punctatus</i>                     | <i>Septoria dracocephali</i>                 |
| * <i>Bouteloua oligostachya</i> (Nutt.) Torr. | * <i>Dulichium arundinaceum</i> (L.) Britton |
| <i>Helminthosporium</i> sp.                   | <i>Puccinia asterum</i>                      |
| <i>Brassica juncea</i> L.                     | <i>Eleocharis palustris</i> (L.) R. & S.     |
| <i>Albugo candida</i>                         | <i>Claviceps nigricans</i>                   |
| <i>Bromus inermis</i> Leyss.                  | <i>Erysiphe polygoni</i> DC.                 |
| <i>Helminthosporium bromi</i>                 | <i>Cicinnobolus cesati</i>                   |
| <i>Calendula officinalis</i> L.               | <i>Euchlaena mexicana</i> Schrad.            |
| <i>Puccinia emiliae</i>                       | <i>Puccinia sorghi</i>                       |
| <i>Carex grisea</i> Wahl.                     | <i>Fragaria</i> sp.                          |
| <i>Cintractia caricis</i>                     | <i>Yellows</i>                               |
| * <i>Carex jamesii</i> Schw.                  | <i>Helianthus annuus</i> L.                  |
| <i>Stagonospora paludosa</i>                  | <i>Septoria helianthe</i>                    |
| <i>Carex pennsylvanica</i> Lam.               | <i>Hordeum jubatum</i> L.                    |
| <i>Stagonospora carcinella</i>                | <i>Ustilago lorentziana</i>                  |
| <i>Catalpa bignonioides</i> Walt.             | <i>Ipomoea hederacea</i> Jacq.               |
| <i>Phyllosticta catalpae</i>                  | <i>Septoria bataticola</i>                   |
| * <i>Cephalanthus occidentalis</i> L.         | <i>Juglans nigra</i> L.                      |
| <i>Puccinia seymouriana</i>                   | <i>Melanconis juglandis</i>                  |
| <i>Chenopodium album</i> L.                   | * <i>Juniperus scopulorum</i> Sarg.          |
| <i>Septoria atriplicis</i>                    | <i>Gymnosporangium globosum</i>              |

- Gymnosporangium juniperi-virginianae*
- \**Lecanium* sp.  
*Cordyceps clavulata*
- \**Leersia oryzoides* (L.) Sw.  
*Cladotrichum leersiae*
- \**Lespedeza violacea* (L.) Pers.  
*Uromyces lespedezae-procumbentis*
- \**Lespedeza virginica* (L.) Britt.  
*Uromyces lespedezae-procumbentis*
- Linum usitatissimum* L.  
*Phlyctaena linicola*
- Lonicera tatarica* var. *alba* Hort.  
*Glomerularia corni*
- \**Luperina stipata* Morr.  
*Cordyceps* sp.
- Lycium halimifolium* Mill.  
*Erysiphe polygoni*
- \**Mentha gentilis* L.  
*Puccinia menthae*
- Mitella diphylla* L.  
*Septoria mitellae*
- \**Mirabilis jalapa* L.  
*Cercospora mirabilis*
- \**Nicotiana affinis* T. Moore  
Mosaic
- Poa pratensis* L.  
*Colletotrichum graminicolum*
- Plantago aristata* Michx.  
*Peronospora plantaginis*
- Podophyllum peltatum* L.  
*Phyllosticta podophylli*
- Populus deltoides* Marsh.  
*Dothichiza populea*
- Populus tremuloides* Michx.  
*Sclerotinia bifrons*
- Prunus persica* L.  
*Phyllosticta persicae*
- Prunus virginiana* L.  
*Podosphaera oxycanthae*
- \**Pyrenanthemum flexuosum* (Walt.) BSP.  
*Puccinia menthae*
- Pyrus communis* L.  
*Gymnosporangium globosum*
- Quercus macrocarpa* Michx.  
*Taphrina coerulescens*
- Quercus prinoides* Willd.  
*Phyllosticta quercus*
- Quercus rubra* L.  
*Armillaria mellea*
- Quercus velutina* Lam.  
*Taphrina coerulescens*
- Ranunculus* sp.  
*Septoria septentrionalis*
- Ranunculus abortivus* L.  
*Botrytis cinerea*
- Ribes* sp.  
*Cronartium ribicola*
- \**Rubus procumbens* Muhl.  
*Phragmidium uredinis*
- Rubus* sp.  
*Pucciniastrum americanum*
- Salix interior* Rowlee  
*Cylindrosporium salicinum*
- Salix nigra* Marsh.  
*Uncinula salicis*
- \**Secotium agaricoides* (Czern.) Hollos  
*Sporodinia grandis*
- Solanum carolinense* L.  
*Puccinia tubulosa*
- Solidago latifolia* L.  
*Septoria virgaurae*
- \**Sonchus arvensis* L.  
*Marssonina sonchi*
- Strophostyles pauciflora* (Bench.) Wats.  
*Phyllosticta phaseolina*
- Tamarix* sp.  
*Botrytis cinerea*
- \**Tulipa* sp.  
*Botrytis tulipae*
- Vigna sinensis* Endl.  
*Amerosporium oeconomium*
- Viola sororia* Willd.  
*Colletotrichum violanum*
- Viola tricolor* var. *hortensis* DC.  
*Cercospora granuliformis*
- Vitis vulpina* L.  
*Discosia artoceras*
- Zinnia elegans* Jacq.  
*Erysiphe cichoracearum*

## LITERATURE CITED

1. ANDERSON, H. W.  
1922. New species of fungi from Illinois. Trans. Ill. Acad. Sci., 15:126-129.
2. ANDERSON, J. P.  
1907. Iowa Erysiphaceae. Proc. Iowa Acad. Sci., 14:15-46.
3. ANGELL, H. R.  
1929. Purple blotch of onion (*Macrosporium porri* Ell.). Jour. Agr. Res., 38:467-487.
4. ARTHUR, J. C.  
1907-27. Uredinales. North America Fl., 7:83-848.
5. ATANASOFF, D.  
1920. Ergot in grains and grasses. U. S. Dept. Agr., Bur. Pl. Indust., Office Cereal Invest. 127 pp. mimeographed MS.



6. ATKINSON, G. F.  
1897. Some fungi from Alabama collected chiefly during the years 1889-1892. Cornell Univ. (Science) Bul. 3:1-50.
7. BRENTZEL, W. E.  
1926. The pasmo disease of flax. Jour. Agr. Res., 32:25-37.
8. BRISLEY, H. R.  
1923. Studies on the blight of cucurbits caused by *Macrosporium cucumerinum* E. and E. Phytopath., 13:199-204.
9. CHUPP, C.  
1925. Manual of vegetable-garden diseases. 647 pp. N. Y.
10. CLEMENTS, F. E., AND R. POUND  
1896. Report on collections made in 1894-95. Bot. Surv. Univ. Nebr. IV. Lincoln, 48 pp.
11. CLINTON, G. P.  
1906. Ustilaginales. N. Amer. Fl., 7:1-82.
12. DAVIS, J. J.  
1915. Notes on parasitic fungi in Wisconsin III. Tran. Wis. Acad. Sci., 18:251-271.
13. \_\_\_\_\_  
1919. Notes on parasitic fungi in Wisconsin IV, V, VI. Trans. Wis. Acad. Sci., 19<sup>2</sup>:671-727.
14. \_\_\_\_\_  
1922. Notes on parasitic fungi in Wisconsin VII. Trans. Wis. Acad. Sci., 20:408-409.
15. \_\_\_\_\_  
1922. Notes on parasitic fungi in Wisconsin VIII. Trans. Wis. Acad. Sci., 20:419-420.
16. \_\_\_\_\_  
1923. Notes on parasitic fungi in Wisconsin XI. Trans. Wis. Acad. Sci., 21:287-302.
17. DEARNESS, J.  
1917. New or noteworthy North American fungi. Mycologia., 9:345-364.
18. \_\_\_\_\_  
1928. New and noteworthy fungi V. Mycologia., 20:233-246.
19. DECKER, G. C.  
1930. The biology of the four-lined borer, *Luperina stipata* (Morr.). Iowa Agr. Exp. Sta. Res. Bul. 143:125-162.
20. DIEDICKE, H.  
1915. Pilze VII. Sphaeropsideae, Melanconieae. Kryptogamenfl. Mark Brandenburg, 9:442.
21. DICKSON, B. T.  
1922. Studies concerning mosaic diseases. MacDonald College Tech. Bul. 2:1-125.
22. DRECHSLER, C.  
1923. Some graminicolous species of *Helminthosporium* I. Jour. Agr. Res. 24:641-740.
23. DURAND, E. J.  
1902. Studies in North American Discomycetes. II. Some new or noteworthy species from central and western New York. Bul. Torrey Bot. Club. 29:458-465.
24. ELLIS, J. B., AND B. M. EVERHART  
1888. New species of fungi from various localities. Jour. Mycol. 4:97-107.

25. ————— AND —————  
1893. New species of North American fungi from various localities. Proc. Acad Nat. Sci. Phila. 1893:128-172.
26. ————— AND E. W. HOLWAY  
1885. New fungi of Iowa. Jour. Mycol. 1:4-6, 31.
27. FARLOW, W. G.  
1883. Enumeration of the Peronosporae of the United States. Bot. Gaz. 8:305-315, 327-357.
28. GAUMANN, E.  
1923. Beitrage zu einer Monographie der Gattung Peronospora Corda. Beitr. Kryptogamenfl. Schweiz. 5:82-83.
29. GILMAN, J. C., AND W. A. ARCHER  
129. The fungi of Iowa parasitic on plants. Iowa State College Jour. of Sci., 3:299-507.
30. GRAVES, A. H.  
1923. The Melanconis disease of the butternut (*Juglans cinerea* L.). Phytopath. 13:411-435.
31. HEDGECOCK, G. C., AND N. R. HUNT  
1916. *Dothichiza populea* in the United States. Mycologia 8:300-308.
32. HOPKINS, E. F.  
1921. The botrytis blight of tulips. Cornell Agr. Exp. Sta. Mem. 45:315-359
33. KAUFFMAN, C. H.  
1918. The Agaricaceae of Michigan. Mich. Geol. and Biol. Surv. Biol. Ser. 5 Publ. 26:1-924.
34. KELLERMAN, W. A.  
1902. Ohio fungi. Fascicle III. Jour. Mycol. 8:5-11.
35. LENDNER, A.  
1908. Les Mucorinees de la Suisse. Beitr. Kryptogamenfl. Schweiz 3:1-180.
36. MARTIN, G.  
1887. Enumeration and description of the Septorias of North America. Jour. Mycol. 3:37-41, 49-53, 61-69, 73-82, 85-94.
37. PECK, C. H.  
1879. Descriptions of 80 new species of fungi. N. Y. State Mus. Nat. Hist. Rept. 32:17-72.
38. PETTIT, R. H.  
1895. Studies in artificial culture of entomogenous fungi. N. Y. (Cornell) Agr. Exp. Sta. Bul. 97.
39. PLAKIDAS, A. G.  
1926. Strawberry "yellows", a degeneration disease of the strawberry. Phytopath. 16:423-426.
40. ROSTOWZEW, S. J.  
1903. Beitrage zur Kenntnis der Peronosporaeen. Flora 92:405-430.
41. SACCARDO, P. A.  
1882-1928. Sylloge Fungorum. Padua. 24 vol.
42. SALMON, E. S.  
1900. A monograph of the Erysiphaceae. Mem. Torrey Bot. Club 9:1-292.
43. SEAVER, F. J.  
1922. Phyllostictales. N. Amer. Fl. 6:1-84.

44. ————— AND P. F. SHOPE  
1930. A mycological foray through the mountains of Colorado, Wyoming and South Dakota. *Mycologia* 22:1-8.
45. STEVENS, F. L.  
1913. The fungi which cause plant disease. 754 pp. N. Y.
46. TAUBENHAUS, J. J.  
1914. Recent studies of some new or little known diseases of the sweet potato. A new leaf spot. *Phytopath.* 4:320.
47. THARP, B. C.  
1917. Texas parasitic fungi. *Mycologia*, 9:105-124.
48. THOM, C., AND M. B. CHURCH  
1926. The aspergilli. Baltimore, pp. 1-272.
49. UNDERWOOD, L. M.  
1897. Some new fungi, chiefly from Alabama. *Bull. Torrey Bot. Club*, 24:81-86.
50. WILCOX, E. M.  
1903. A leaf curl disease of oaks. *Alabama Agr. Exp. Sta. Bul.* 126:169-187.
51. WILSON, G. W.  
1907. Studies in North American Peronosporales I. The genus *Albugo*. *Bull. Torrey Bot. Club*, 34:61-84.
52. —————  
1914. The identity of the anthracnose of grasses in the United States. *Phytopath.* 4:106-112.
53. —————  
1930. Noteworthy Iowa fungi. *Proc. Iowa Acad. Sci.*, 37:111-114.
54. WINTER, G.  
1883. New North American fungi. *Bull. Torrey Bot. Club*, 10:49-50.
55. YOUNG, P. A., AND H. E. MORRIS  
1927. Sclerotinia wilt of sunflowers. *Montana Agr. Exp. Sta. Bul.* 208:3-32.





## A STUDY OF CARAMEL COLOR<sup>1</sup>

JOHN B. SHUMAKER WITH J. H. BUCHANAN

*From the Department of Chemistry, Iowa State College*

Accepted for publication May 25, 1932

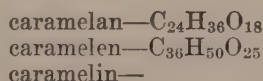
Caramel has been used as a coloring material in foods and beverages for many years. The quality of caramels, however, is quite variable, depending upon the material from which they are made, the method of manufacture, and the nature of the products in which they are to be used as a color.

The coloring powers of caramels vary considerably, even though they may be prepared from the same carbohydrate. A given caramel may show stability at one H-ion concentration, and separate from solution at another H-ion concentration. These facts indicate that caramels may be colloidal in nature and that there is a definite isoelectric point for the different varieties of caramel.

As early as 1838, Peligot (7) examined the products of the dry distillation of sugars. Gélis (4) in 1858, and Stolle (11) in 1899, also did much to establish the nature of the distillates. Sangiori (10) reported the presence in caramel of furfural, acetone, formaldehyde, formic acid, and acetic acid, all in small quantities. By far the greater portion of the distillate is water.

This leads one to believe that the principal reaction promoted by heating sugars is that of dehydration and that other products are formed to a small extent at a temperature slightly above the melting point of sugar.

Gélis (4) also showed that sucrose, when heated at temperatures around 200°C., was converted progressively into a number of dehydration products by successive dehydration. These products he named in the order of their formation:



Caramelin was a more highly dehydrated product with a rather uncertain formula but decidedly colloidal.

Graham (5) showed that caramelan and caramelen dialyzed readily and that caramelin did not. Cunningham and Dorée (3) prepared caramelan in nearly pure form by heating sucrose at 170°-180°C. until the loss in weight was 12 per cent. After further purification from alcoholic ammonia they were able to verify the formula of Gélis for caramelan by freezing point methods.

Pictet and Andrianoff (8) working at 10-15 mm. pressure at 185°-190°C., were able to produce the entire series including isosaccharosan, C<sub>12</sub>H<sub>20</sub>O<sub>10</sub>. This compound is obtained by simple loss of a molecule of water and is nearly colorless. Further dehydration takes place between molecules, forming larger molecules in multiples of C<sub>12</sub>.

<sup>1</sup>From a thesis submitted to the Graduate Faculty of Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Ripp (9) prepared caramelan from levulose, proving that sucrose is not the only carbohydrate which will yield caramelan.

Beal and Bowey (1) prepared caramel from glucose with the aid of catalysts such as ammonia, ammonium chloride, sodium carbonate, hydrochloric acid and ammonium sulfate. They showed that with higher temperature and longer heating, caramels of higher coloring power and of less stability were obtained. Beal and Applegate (2) showed that sucrose caramels were equal to or better than glucose caramels.

In order to study caramel more closely and to correlate the colloidal properties with the chemical compounds obtained by previous workers, three series of caramels were prepared from sucrose at temperatures of 190°, 200°, and 210°C. Certain representative caramels from each series were subjected to various H-ion concentrations, to dialysis, and to electrophoresis. The colloidal properties of the compounds of caramel are clearly shown to be related to the temperature and time of heating of the carbohydrate.

#### PREPARATION OF CAMELANS

A number of caramels were prepared at different temperatures and with varying intervals of time of heating, without the aid of a catalyst. In order to make a systematic comparison of caramels prepared at a given temperature, the loss in weight during a definite interval of heating was taken as a criterion. The small amounts of substances other than water evolved were considered as negligible, and the loss in weight of the sucrose upon heating was considered as water.

Dry sucrose of a high grade was the carbohydrate employed in the experiments. Charges of two hundred grams of sucrose were used. These were weighed upon a trip balance with an accuracy of 0.1 gram.

It was noted in all previous work that little attention was given to accurate temperature control. Since a given charge of sucrose was to be heated at temperatures of 190° to 200°C. over periods of time ranging from 30 minutes to 130 minutes, a wide variation in weights would result in the final products. Obviously it would be nearly impossible to obtain results which could be accurately duplicated. After some preliminary experiments with various methods of heating, this was found to be the case.

An electrically heated device provided with mechanical stirrers was constructed which gave satisfactory results as is indicated in table 1. These runs are typical of the routine runs which are recorded in tables 2, 3 and 4. The duplicate results (b) check the original runs (a) closely, giving rise to an experimental error of about 0.2-0.3 per cent.

TABLE 1. *Influence of time and temperature on loss in weight*

Series No.	Time min.	Temp.	Loss percentage		
			a	b	average
A-1	40	190	0.5	0.4	0.45
A-3	60	190	5.9	5.8	5.85
B-1	30	200	2.3	2.3	2.30
B-2	35	200	4.45	4.45	4.45
B-3	40	200	5.85	6.05	5.95
B-4	45	200	7.85	7.25	7.75
B-5	50	200	8.40	8.25	8.30
C-1	20	210	2.95	2.85	2.90

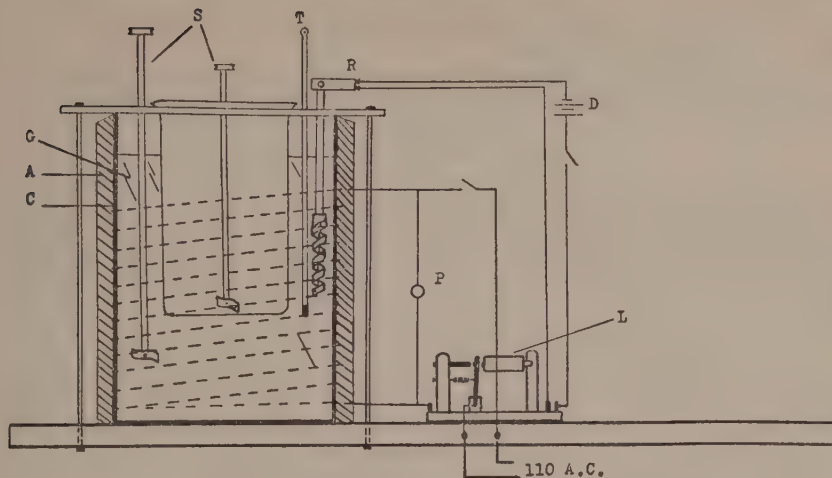


Fig. 1. Design of electric heater for constant temperature bath

#### DESCRIPTION OF APPARATUS

Figure 1 is a design of the construction of the electric heater. The bath itself is an aluminum cylindrical vessel around which an insulating layer of asbestos paper is closely wrapped. Around the asbestos layer there is placed a coil of twenty turns of No. 18 electric resistance wire. The ends are connected to an electric current. The wire is held in place firmly by a thick layer of fire cement.

A square plate of asbestos board covers the top. Four bolts pass through the corners and into the base upon which the apparatus rests. These bolts serve to hold the bath in a rigid position upon the base. A large hole is cut in the center of the plate of such size that a low-form Griffin Pyrex beaker may be suspended in it and supported only by its rim.

Distributed about the large hole are three small holes. Into one of these is inserted the bath stirrer (S) which keeps the entire bath at uniform temperature by forced circulation. Into another is inserted a thermometer (T) which has been accurately calibrated at the temperature at which the bath is operated. Through the third hole is inserted a regulating device (R) which, by expansion of the metal coil at its lower end, causes the heating current to break when the proper temperature has been reached.

At the top of the regulator are two contact points which, when they touch, close a circuit through the two dry cells (D). This current passes through the magnet on the relay (L). The magnet draws the small vertical arm toward it and causes a break in the 110 volt heating circuit, and the bath ceases to heat. When the temperature of the bath drops sufficiently, the regulator releases the vertical bar, which is pulled over by a small spring and contact is again made in the heating circuit. A pilot light (P), placed across the terminals of the heating unit, indicates when the bath is heating.

The bath medium chosen was glycerin. It becomes very fluid at high temperatures, circulates readily, and has the advantage over oil in that it

can be washed conveniently from the surface of the beaker with water after the completion of a run.

With the device as described, a temperature of 200°C. was evenly maintained over a period of time with a fluctuation of 0.1 to 0.2°C.

#### METHOD OF PROCEDURE

Into a one-liter beaker, which has been previously described, was carefully weighed two hundred grams of dry sucrose. Both the weight of the empty beaker and of the beaker containing sucrose were recorded. Meanwhile the bath was heated to the desired temperature. The beaker was then placed in the bath, clamped firmly into position, and the time quickly noted.

In a short while the sucrose began to melt and to color slightly. After complete melting had taken place, a slight foaming began to occur which increased rapidly until the foam began to fill the beaker. Rapid stirring at this point with the mechanical stirrer broke the foam sufficiently to keep it well within the beaker. Within a comparatively short time the foaming decreased, indicating that the velocity of the reaction was not so great. As foaming decreased, the color became correspondingly darker. After an interval of time, if the run was carried on for a sufficient period, the foaming ceased, and the mass became quite viscous.

At this point a second stage of foaming began. The mass being very viscous by this time, it was increasingly difficult to stir successfully with the mechanical stirrer. From this point on, stirring by hand became necessary, in order to prevent the very dark viscous mass from rising over the top of the beaker. The velocity of this second reaction slowly decreased and the mass gradually thickened until it was evident that it would be useless to continue the run at the stated temperature.

When the time allotted for the run had expired, the beaker was quickly removed from the bath, the glycerin washed from the outside, and when it had cooled sufficiently, the beaker was weighed. The loss in weight was recorded, as was also the percentage loss in weight.

As soon as one run was completed, a second was immediately started. In this way a series of runs was made at a given temperature, each run being heated a definite number of minutes longer than the previous one.

In this manner, three series of caramels were prepared at temperatures of 190°, 200°, and 210°C. By plotting the time of heating against the percentage loss in weight a number of interesting observations were made and conclusions drawn. Tables 2, 3 and 4 represent the essential data collected for the production of the curves in figure 2.

TABLE 2. *Series A. Caramels prepared at bath temperature 190°C.*

Run no.	Time min.	Loss p'c't'g.	Run no.	Time min.	Loss p'c't'g.
1	40	00.45	7	100	09.4
2	50	3.05	8	110	10.45
3	60	5.85	9	120	10.6
4	70	7.2	10	130	11.3
5	80	8.45	11	140	12.05
6	90	8.7	12	150	11.95



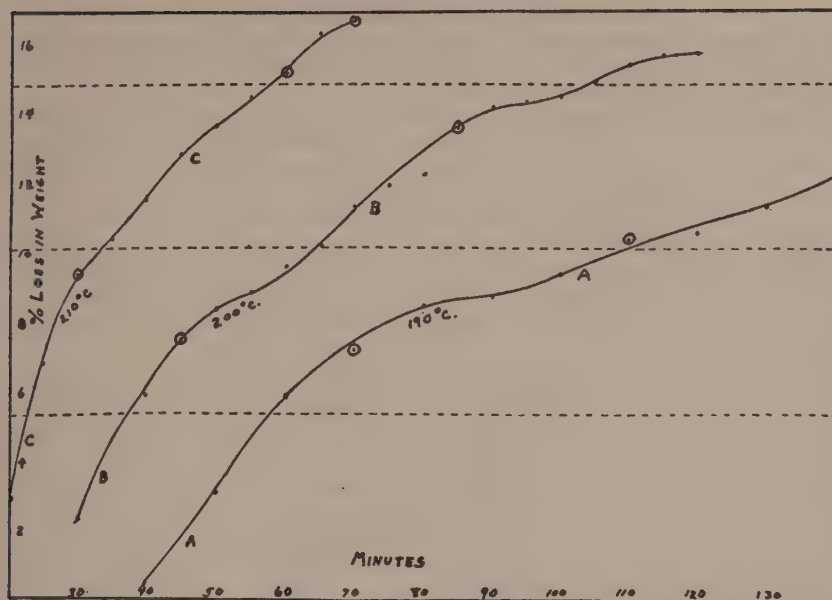


Fig. 2. Curves showing loss in weight during lapse of time of heating

TABLE 3. *Series B. Caramels prepared at bath temperature 200°C.*

Run no.	Time min.	Loss p'c't'g.	Run no.	Time min.	Loss p'c't'g.
1	30	2.3	11	80	12.1
2	35	4.45	12	85	13.65
3	40	5.9	13	90	14.15
4	45	7.55	14	95	14.25
5	50	8.3	15	100	14.50
6	55	8.9	16	105	14.9
7	60	9.6	17	110	15.4
8	65	10.2	18	115	15.65
9	70	11.3	19	120	15.75
10	75	11.9			

TABLE 4. *Series C. Caramels prepared at bath temperature 210°C.*

Run no.	Time min.	Loss p'c't'g.	Run no.	Time min.	Loss p'c't'g.
1	20	2.9	7	50	13.65
2	25	6.85	8	55	14.4
3	30	9.4	9	60	15.15
4	35	10.3	10	65	16.3
5	40	11.5	11	70	16.65
6	45	12.85			

## SERIES A. CARAMELS PREPARED AT 190°C.

In order to clarify the description of the general procedure, it is necessary at this point to discuss in detail the production of the caramels at each of the temperatures mentioned.

For each successive run at 190°, the time of heating was extended by ten minutes. The temperature was kept constant at all times to within 0.1° of 190°. At approximately 30 minutes from the time the beaker was placed in the bath, the sugar had completely melted.

There was no foaming until 40 minutes had elapsed. Suddenly foaming started. By the time 80 minutes had passed, the foaming had nearly ceased and the sucrose was merely a dark pasty mass which could be stirred only with difficulty. The color after melting was light brown, but as foaming continued the brown became more intense until, at 80 minutes, it was quite dark.

When 80 minutes of heating had elapsed, the second stage of foaming began. The vapors became more acrid and penetrating to the nostrils. The second stage of foaming was not so violent as the first, indicating that dehydrating was not proceeding so rapidly as before.

The color now became very dark, nearly black; the mass became so viscous that it had to be stirred by hand. From the color it was evident that, with prolonged heating, decomposition products other than water were forming in increasing quantity. After 140 minutes had passed it was considered impractical to continue the run longer.

The above description is that of the longest run made at 190°. All of the shorter runs behaved in an exactly similar manner up to the time allotted to them, when they were removed from the bath.

## SERIES B. CARAMELS PREPARED AT 200°C.

At this higher temperature it was to be expected that the sucrose would melt more rapidly, and that the time of heating would be materially shortened in order to form the same caramels which had been produced in series A.

This was found to be the case. The sucrose became completely melted in about 20 minutes and foaming began immediately. The second stage of foaming began in 35 minutes. The third occurred at 55 minutes. A caramel of series B produces much darker solutions than one of series A which had been prepared with the same time of heating.

## SERIES C. CARAMELS PREPARED AT 210°C.

The same general observations were made as in series A and B. However, as was to be expected, complete melting had occurred within 15 minutes, and foaming began at once. The second foaming stage began in 19 minutes and the third in 30 minutes.

The data for these three series are recorded in tables 2, 3 and 4. These data were plotted on coordinate paper (fig. 2). With the aid of the curves important deductions were made which are not obvious from the data. In the graph (fig. 1) the loss in weight of the members of each series is plotted against the time of heating in minutes. Three dotted horizontal lines located respectively at 5.23 per cent, 10.52 per cent and 14.03 per cent are included to indicate the points at which isosaccharosan, caramelan, and caramelen should be formed.

In order to prepare isosaccharose ( $C_{12}H_{20}O_{10}$ ), one molecule of sucrose must lose one molecule of water. The weight of water lost would be 5.23 per cent of the molecular weight of sucrose.

The formation of caramelan from sucrose requires the loss of four molecules of water from two molecules of sucrose. The loss in weight required is 10.52 per cent.

Caramelan is formed from three molecules of sucrose by the loss of eight molecules of water. In this case the loss is 14.03 per cent.

These values are theoretical and cannot be attained practically for the reason that other products are formed at the same time to a small extent. Therefore, in order to prepare the purest caramelan that is possible, it would be necessary to heat sucrose until the loss in weight was somewhat over 10.52 per cent. Cunningham and Dorée (3) obtained almost pure caramelan by heating sucrose until the loss was 12 per cent.

Foaming of the caramel as it is heated is indicative of the chemical reaction whereby water is lost. Excessive foaming indicates rapid loss of water, whereas, little foaming indicates little loss. The greatest foaming occurs at the beginning of a definite chemical reaction and at the end there is practically no foaming.

Therefore, in the study of the curves, the horizontal portions indicate the end of one definite dehydration reaction. The rising portion immediately following indicates the beginning of a new reaction.

*Curve A.* The running temperature of  $190^{\circ}$  was selected as being the lowest practical temperature at which caramel could be prepared within a reasonable length of time. The melting point of sucrose is approximately  $10^{\circ}$  below this.

The first stage of foaming started within 40 minutes and had become essentially complete at the 80 minute interval. No decided break can be seen in this portion of the curve. A break should appear in the region of 5 per cent loss, but the relatively low temperature causes the merging of the two reactions into practically one. No foaming stage was noted again until the 80 minute interval was reached. Here a decided rise in the curve is again noticed, showing the completion of the third reaction and the beginning of the fourth.

The first stage should indicate the formation of isosaccharosan, but because of the low temperature, the reaction is not rapid enough to show a distinct break. Consequently, the beginning of the second stage, or the formation of caramelan, is not discernable. The second stage as shown is then really the third stage, and caramelan begins to form within the 80 minute interval.

The loss should be 10.52 per cent, but the curve indicates that caramelan has been formed when the loss is only about 8.5-9 per cent. In order to account for this apparent discrepancy the temperature and viscosity of the caramel must be taken into account. The viscosity is continually increasing while the temperature remains at  $190^{\circ}$ .

As viscosity increases it becomes more and more difficult to remove the water as rapidly as it is formed, by stirring. Consequently, although the water may be completely liberated, some of it is retained mechanically for a short time before it can be vaporized. According to the curve, when caramelan has been completely formed, there is still mechanically retained as much as 1-1.5 per cent of unvaporized water. The result is that caramelan has been apparently formed with a loss of only about 9 per cent water instead of 10.52 per cent.

At a higher temperature, water would be eliminated more rapidly as it is formed. On curves B and C this is found to be true.

A slight rise in the curve is again noticeable at the 140 minute interval, although no foaming was observed. It was considered impractical to continue this curve further because of the fact that the mass became un-stirrable. At higher temperatures the mass remains liquid until the loss is greater.

At the temperature of  $190^{\circ}$ , the formation of isosaccharosan cannot be detected. The formation of caramelan is readily observed, however, with a loss of weight about one per cent below the theoretical, which has been explained. Caramelan cannot be prepared satisfactorily at this temperature.

*Curve B.* Foaming begins in about 20 minutes. At the 35 minute interval a slight break occurs which may be construed as the completion of the reaction, sucrose = isosaccharose. The break occurs at the proper point for the formation of isosaccharose, but is not definite, probably because the temperature is high, causing the rapid elimination of water. The second break indicates quite clearly the complete formation of caramelan, and the beginning of formation of caramelen. Here, as in curve A, caramelan is formed with a loss of water about one per cent below the theoretical.

A small break is again observed at the 95 minute interval, although no unusual foaming was observed. The break occurs very close to the 10.52 per cent line, which is theoretical for caramelen. Assuming the mechanical retention of water to be roughly the same, the close agreement between the theoretical loss and the experimental loss can be accounted for by the increase in other decomposition products which vaporize with the water. The higher temperature would favor the formation of substances other than water.

This break shows quite clearly the formation of three dehydration products. Isosaccharosan is formed at the proper place. Caramelan formation is indicated quite distinctly with a loss about one per cent below the theoretical. Caramelen appears to be formed at the proper place also. There is probably mechanical retention of some water, but this is offset by the formation of more decomposition products at the higher temperature.

*Curve C.* In this case three distinct stages of foaming were observed. The high temperature of  $210^{\circ}$  caused foaming to occur at 15 minutes and again at 19 minutes. However, water was eliminated so rapidly that it was impossible to record the weights in such a way as to show a break on the curve.

At 30 minutes the third stage began. A distinct break occurs here at the 10.52 per cent line. It is probable that at this high temperature water is driven off almost as rapidly as it is formed and the result is that the loss is almost theoretical for that required to form caramelan.

Another depression is to be noted at the 55 minute interval, but no specific foaming was noted at this stage. This depression is indicative of the complete formation of caramelen.

A consideration of all three curves brings out the following observations:

1. Curve B only, indicates the formation of isosaccharosan.
2. All three curves show clearly the formation of caramelan at or near the theoretical point.



- . Curves B and C show evidence of the formation of caramelen. Curve A cannot be carried far enough to show a similar point.
4. Variations from the theoretical losses can be explained on a basis of mechanical retention of water due to relative temperature and viscosity.

#### STUDY OF COLLOIDAL PROPERTIES OF CARAMELS

In order to study the colloidal properties, certain caramels were selected from each series of runs which most closely represented the compounds isosaccharosan, caramelan, and caramelen. The caramels selected were as follows:

1. Caramels on curve A which had been formed by heating 70 and 110 minutes, respectively. These will be designated hereafter as A-70 and A-110.
2. Caramels on curve B—B-45 and B-85.
3. Caramels on curve C—C-30, C-60, and C-70. C-70 was selected because it had undergone the greatest loss at the highest temperature employed, and therefore represented the most highly caramelized product obtained.

*Dialysis.* No attempt was made to establish any quantitative relationships in the experiments on dialysis. Collodion membranes were prepared by allowing a film to dry upon the inner surface of a large test tube. These were easily removed from the tube by soaking in water.

Solutions of the selected caramels were made by dissolving a small quantity of the caramel in distilled water until the color was an intense brown. The solutions were still dilute with respect to the quantity of caramel present.

Each of the seven selected caramels were placed in separate collodion bags. Each bag was filled and then suspended in a two-liter beaker containing distilled water. Diffusion began immediately as indicated by the color of the distilled water in the beaker. The water was changed every two hours.

Within 6 hours solutions of A-110, B-85, and C-60 had ceased to dialyze. The solutions inside the bags were almost as dark in color as they originally had been. Apparently most of the caramel in each of these bags did not diffuse through the membrane.

After 24 hours, A-70, B-45, and C-30 had practically ceased diffusing. By the color of the contents of the bags, it was concluded that there was some colloidal material present. However, most of the contents were crystalloid because so much time was required to remove it by dialysis.

In the higher portions of the curves the caramels are shown to be quite colloidal in nature, while the lower caramels are shown to be mostly crystalloidal.

*Electrophoresis.* According to Holmes (6) the charge of electricity on a colloidal particle in suspension is caused by the preferential absorption of positive or of negative ions from the solution on the surface of the particle. A particle thus charged, on electrolysis, will move toward the electrode of opposite sign. Caramels are no exception to this rule.

To demonstrate the mobility of caramel on electrolysis, an apparatus similar to that described by Holmes (6) was constructed. A layer of clear distilled water was carefully superimposed on the caramel solution, the platinum electrodes were immersed in the water layer, and a direct current was passed through at 110 volts for 60 minutes.

The distance which the colored layer at the negative electrode moved downward was carefully measured and noted in table 5.

TABLE 5. *The downward movement of the layer at the negative electrode*

Caramel sol.	mm. lowering	Caramel sol.	mm. lowering
A-70	1.70	A-110	3.55
B-45	0.85	B-85	1.40
C-30	0.40	C-60	5.10
		C-70	1.00

Caramels on the lower portions of the curves (A-70, B-45, and C-30) undergo electrophoresis to a less extent than the upper members. This fact would indicate that the charge upon the lower members is not so great as that on the higher members. The lower caramels would be considered either as particles which are near their isoelectric point, or as particles which border on true solution particles in size. The results of dialysis favor the idea that the particles are near in size to particles in true solution.

*Effect of H-ion Concentration.* From the results of dialysis and cataphoresis it is concluded that the caramels which have been prepared are electro-negatively charged colloids. Whatever the source of the charge upon the particles, whether it is merely a difference of potential set up between the particles and the medium, or whether it is a case of preferential absorption of hydroxyl ions, the addition of ions bearing opposite charges should have a neutralizing effect upon the charges already present on the particles.

Assuming that hydroxyl ions are absorbed to a greater extent than hydrogen ions, the colloid becomes negatively charged. Addition of acids, or in other words, addition of hydrogen ions, would tend to neutralize the negative charges and eventually cause the particle to become electrically neutral. At the neutral zone coagulation and precipitation of the particles usually occur and the isoelectric point is said to have been reached.

Acids which are highly ionized would be expected to have a greater precipitating effect upon caramel than slightly ionized acids of equivalent strengths. In order to avoid incorrect conclusions, it would be best to choose acid solutions which contained equal molar concentrations of hydrogen ions from different acids.

For the purposes of the experiment, the acids citric, phosphoric and sulfuric were selected. One of these acids represents the weak organic acids which are used in acid beverages, and one a weak inorganic acid (phosphoric), and one the moderately strong inorganic acid (sulfuric).

In order to secure comparative results as far as H-ion concentrations were concerned, solutions of each acid were prepared having the same approximate pH, namely, 3, 2, and 1.5. These solutions were accurately standardized by the electrometric method, using the hydrogen electrode. Table 6 is designed to give the accurate pH of the solutions mentioned in

the foregoing. For the purposes of discussion, the approximate values will be used.

TABLE 6. *The pH of acids*

Approx. pH	Citric acid	Phosphoric acid	Sulfuric acid
3			2.90
2	1.97	2.00	2.17
1.5	1.46	1.49	1.56

Since a rise in temperature causes a change in the pH, increasing the degree of ionization of the acid, the effect of both the hot and cold acids on the caramels was determined. The procedure is as follows:

To 50cc. of citric acid, pH-1.5, was added a concentrated solution of caramel until a very deep but still transparent color was obtained. Only a few drops of the caramel solution were required to produce the proper color, and the change in pH by the addition of so small a quantity was considered negligible. The flask was then corked carefully to exclude dust and to prevent evaporation, and set aside in diffused daylight for observation.

The limit of time set for observation was two weeks. If precipitation did not occur within that time the caramel was considered stable toward citric acid of pH 1.5. This procedure was carried out in the same way for the other acid solutions previously described—seven in all. This test constitutes the "cold test" of the different acids on the seven caramels which had been selected for examination of their colloidal behavior.

The "hot test" was conducted in a somewhat similar way. The caramel was added to the acid solution in a small flask. The solution was then boiled gently for a period of twenty minutes, tightly stoppered while still boiling hot, and set aside for observation.

In this way the effect of each acid upon each caramel was determined. The results of these tests are recorded as follows:

TABLE 7. *Effect of cold acids on caramels*

Series no.	Approx. comp.	Time required for caramel to ppt.					
		Citric 2	pH. 1.5	Phosphoric 2      1.5	pH. 3	Sul- furic 2	pH. 1.5
A-70	Isosaccharosan	—	—	—	—	—	—
B-45	"	—	—	—	—	—	—
C-30	"	—	—	—	—	—	—
A-110	Caramelan	—	—	—	—	—	11d
B-85	"	—	—	—	—	—	11d
C-60	"	—	—	—	6d	—	75m
C-70	Caramelen	—	—	6d	1m	6d	1m

d = days, h = hours, m = minutes.

— indicates no apparent precipitate found within two weeks.

TABLE 8. *Effect of hot acids on caramels*

Series no.	Approx. comp.	Time required for caramel to ppt.					
		Citric 2	pH. 1.5	Phosphoric 2 1.5		pH. 3	Sulfuric 2 pH. 1.5
A-70	Isosaccharosan	—	—	—	—	—	6d
B-45	"	—	—	—	—	—	6d
C-30	"	—	—	—	—	—	3h
A-110	Caramelan	—	—	—	6d	—	60m
B-85	"	—	—	—	?	—	75m
C-60	"	—	—	—	6d	—	60m
C-70	"	—	—	—	1m	—	30m

d = days, h = hours, m = minutes.

— indicates no apparent precipitate found within two weeks.

Considering the tables given it is found that cold citric acid, phosphoric and sulfuric acids have no precipitating effect upon the caramels A-70, B-45 and C-30 which represent isosaccharosan on the curves (fig. 1), in two weeks time. From the results of dialysis and electrophoresis, caramels in this region have been shown to be only partly colloidal.

These caramels were found to dialyze much more than the higher caramels and also migrated a shorter distance when subjected to the action of the electric current. When subjected to acid solutions ranging in pH from 1.5 to 2 and 3, there was no precipitation in the cold.

Sulfuric acid with pH 1.5 when hot caused precipitation after an interval of six days had elapsed. In the case of C-30, precipitation occurred much sooner, because C-30 represents a caramel formed by a slightly greater loss of weight, and therefore, is somewhat more colloidal in nature than A-70 or B-45.

The caramels A-110, B-85 and C-60, which represent caramelan on the curves, are not affected by citric either hot or cold. However, cold phosphoric acid (pH 1.5) affects C-60 while hot phosphoric (pH 1.5) affects all the caramels. Sulfuric acids with pH 2 and 9 do not affect these caramels when either hot or cold.

C-70, representing caramelen, is not affected by citric acid, but phosphoric (pH 1.5) and sulfuric (pH 1.5) both precipitate it quickly.

The two mineral acids, phosphoric and sulfuric, are more effective precipitating agents than the organic citric acid. When the mineral acids are hot they are more effective than when cold.

#### SUMMARY

1. Three series of caramels have been prepared, all of which, when loss of weight is plotted against time of heating, show the same general regions in which definite compounds form. These regions correspond in general to the requirements for formation of isosaccharosan, caramelan and caramelen.



2. Caramels of low molecular weight are shown to possess low color value, and to dialyze largely. The higher caramels are shown to have opposite properties and are clearly colloidal. All caramels behave as electro-negative colloids under electrophoresis.

3. The effect of H-ion concentrations may be summarized as follows:

- a. The caramels do not precipitate in citric acid solutions having pH 2 and 1.5 within two weeks.
- b. The lower caramels are stable to cold sulfuric acid at pH of 1.5, but are precipitated by the same acid when hot.
- c. Phosphoric acid, pH 2 and sulfuric acid, pH 3 and 2 have no effect either hot or cold.
- d. The caramels are most stable in the presence of citrate ions and less stable in the presence of sulfate ions. Phosphate ions have intermediate precipitating action.

#### LITERATURE CITED

1. BEAL, G. D., AND D. F. BOWEY  
1923. The preparation of acid-fast caramels. *Jour. Pharm. Assoc.*, **12**:405,410.
2. ———— AND GLADYS APPELGATE  
1923. Preparation of acid-fast caramels. II. The preparation of sucrose caramel. *Jour. Pharm. Assoc.*, **12**:850-853.
3. CUNNINGHAM, MARY, AND CHARLES DORÉE  
1917. Contributions to the chemistry of caramel. *Jour. Chem. Soc.*, **111**:589-608.
4. GÉLIS, M. A.  
1858. Action de la Chaleur sur les Substances Neutres Organiques; Étude du Caramel et des Produits Torréfiés. *Ann. Chim. Phys.* (3) **52**:352.
5. GRAHAM, THOMAS  
1862. On liquid diffusion applied to dialysis. *Jour. Chem. Soc.*, **15**:258.
6. HOLMES, HARRY N.  
1928. Laboratory manual of colloid chemistry. John Wiley and Sons, New York, p. 46-49.
7. PELIGOT, EUGENE  
1838. Recherches sur la Nature et les propriétés Chimiques des Sucres. *Ann. Chim. Phys.*, (2) **67**:172-177.
8. PICTET, AMÉ ET N. ANDRIANOFF  
1924. De l'Action de la Chaleur sur le Saccharose. *Helv. Chim. Acta.*, **7**:703-707.
9. RIPP, B.  
1926. Formation of caramel substances in the presence of nitrogenous compounds. *Z. Ver. deut. Zucker-Ind.*, **76**:627-655. Original not seen. Abstracted in *Chemical Abstracts* **21**:1567. 1927.
10. SANGIORI, GIUSEPPE  
0000. Caramelization of sugar and commercial caramel. *Giorn. farm. chim.* **62**:256-261. Original not seen. Abstracted in *Chem. Abst.* **8**:1682. 1914.
11. STOLLE, F.  
1899. Untersuchungen über Karamelkörper. *Z. Ver. Rübenzuck-Ind.*, p. 800-807. Original not seen. Abstracted in *Chem. Zentr.*, p. 1021-1022. 1899. II.



# CONJUGATED SYSTEMS IN FURAN TYPES

HENRY GILMAN AND JOSEPH B. DICKEY

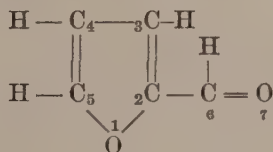
*From the Chemical Laboratory of Iowa State College*

Accepted for publication May 28, 1932

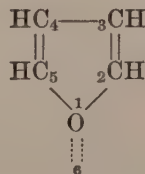
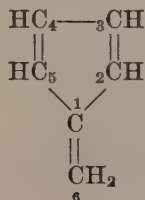
## INTRODUCTION

The pronounced and almost extraordinary tendency of many furan types to undergo nuclear substitution reactions indicates that these heterocycles have super-aromatic characteristics. Current explanations of nuclear substitutions, in general, turn on the preliminary formation of addition compounds. Because of the marked tendency of conjugated systems to undergo addition, it was advisable to determine whether furan types have more active conjugated systems than other related cycles like benzene, and also which conjugated systems are involved in the addition reactions.

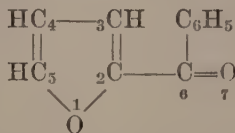
The compounds selected were those having lateral unsaturation which might be considered as forming conjugated systems with units of nuclear unsaturation. Furfural, for example, may be considered to have a multiple series of conjugated systems.



Such systems are (2, 3, 4, 5) and (3, 2, 6, 7); and a 1,6-addition system is (5, 4, 3, 2, 6, 7). Also, if we assume that the oxygen is capable of functioning, latently or otherwise, in an oxonium form, then we have a hypothetical double bond emerging from this oxygen which would give rise to two other conjugated systems: namely, (1, 5, 4) and (1, 2, 3). This latter consideration is highly attractive because it would give a system of crossed conjugated linkages which generally are distinctly more active than a simple conjugated system just as a simple conjugated system is more active than a non-conjugated system or a simple unsaturated unit like an olefinic linkage or a carbonyl group. An illustration would be the comparison of a highly active crossed conjugated system like fulvene with the related furan,



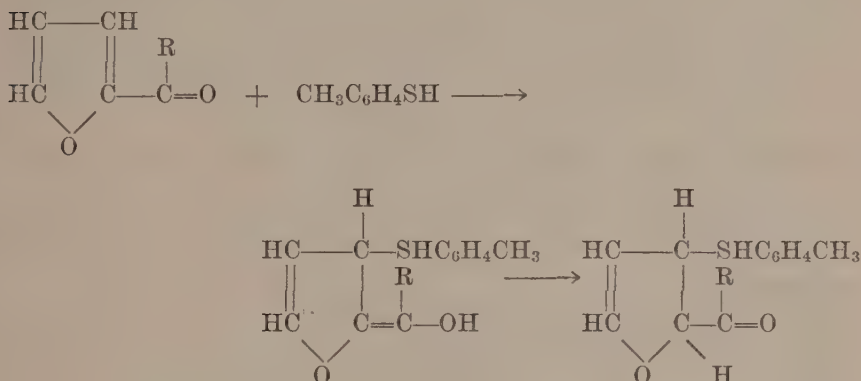
the partial valences emerging from the furan oxygen might then give rise to the crossed conjugated systems (6, 1, 2, 3) and (6, 1, 5, 4). The related



<sup>2</sup>Gilman and Wright, *J. Am. Chem. Soc.*, **52**, 3349 (1930); Freure and Johnson, *ibid.*, **53**, 1142 (1931).



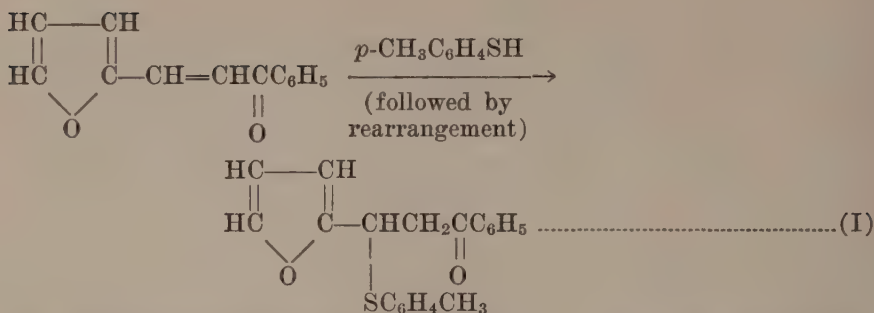
these types were treated with *p*-toluenesulfinic acid ( $p\text{-CH}_3\text{C}_6\text{H}_4\text{SO}_2\text{H}$ ), *p*-thiocresol ( $p\text{-CH}_3\text{C}_6\text{H}_4\text{SH}$ ) and *p*-thiocresylmagnesium iodide ( $p\text{-CH}_3\text{C}_6\text{H}_4\text{SMgI}$ ). These reagents are indicated for such a purpose, having been used effectively for related conjugated systems. If *p*-thiocresol added to such a system one might expect the following transformations, where R is (H) or ( $\text{C}_6\text{H}_5$ ).



The net result would be, as with such systems, addition to the (2,3)-linkage. However, the very high recovery of reactants proves that either no such addition occurred or if it did take place it was followed by 1,4-elimination to give the original compounds. The latter possibility is somewhat remote in view of earlier studies with related conjugated systems. Accordingly, it is reasonable to conclude that the addition reactions which precede nuclear substitution of such furan types do not proceed through the conjugated system (3, 2, 6, 7) and are very probably confined to the (2, 3, 4, 5) conjugated system. Likewise, one can conclude that there is no 1,6-addition to the system (5, 4, 3, 2, 6, 7). This type of 1,6-addition would be necessary, rearrangements excluded, to account for the fact that nuclear substituents are almost invariably found on the *alpha* or 2- or 5-carbon atoms if either *alpha*-position is available, and that direct *beta* or 3- or 4-carbon atom substitution practically never occurs. It should be emphasized here that the related phenyl compounds also do not undergo such addition reactions to a nuclear-lateral conjugated system. However, addition of this kind has been noted in the reaction between highly phenylated compounds and the Grignard reagent<sup>3</sup>.

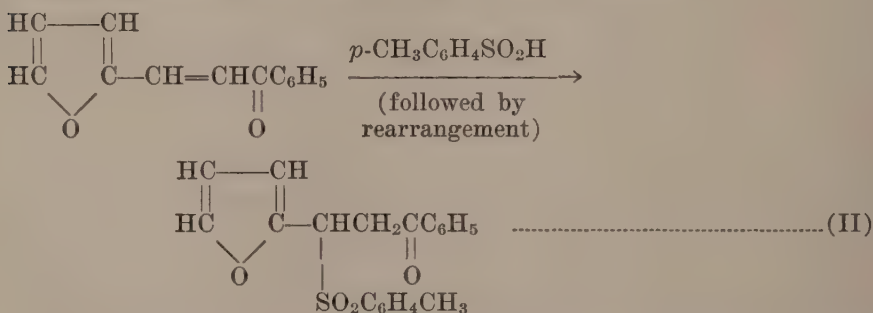
In order to establish with some certainty that the reagents employed for the above-mentioned purpose are reliable for furan compounds having a conjugated system which is entirely lateral and not a mixed nuclear-lateral conjugated system (as with furfural), the same reactions were carried out with compounds like furfuralacetophenone:

<sup>3</sup>Gilman, Kirby and Kinney, *J. Am. Chem. Soc.*, **51**, 2252 (1929); Kohler and Nygaard, *ibid.*, **52**, 4128 (1930).



The lateral conjugated system ( $\text{---CH=CH---C---C}_6\text{H}_5$ ) did undergo reac-

tion. Also, reaction took place with *p*-toluenesulfinic acid:



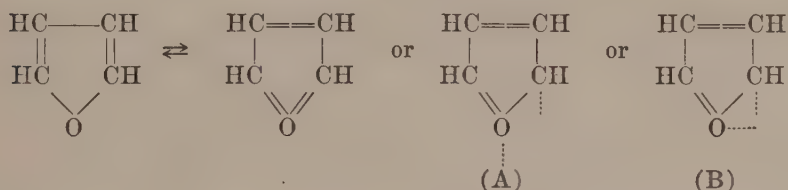
Similar reactions have been studied by numerous investigators<sup>4</sup> using benzene but not furan types.

These studies on lateral conjugated furan types revealed some interesting differences from the related phenyl types, and so make it necessary to proceed with caution in drawing too broad generalizations between such aryl types. First, in contrast with the ready addition of *p*-thiocresylmagnesium iodide to benzalacetophenone<sup>4d</sup>, negative results were obtained with furfuralacetophenone. Only extensive refluxing with furfuralacetophenone gave a small quantity of the addition compound, and there is always the possibility that this very limited reaction took place with *p*-thiocresol formed by the hydrolysis of a small quantity of *p*-thiocresylmagnesium iodide, despite precautions observed to exclude moisture. Second, no condensation was effected with *p*-thiocresol and furylacrylic acid, although such condensation has been noted with the related cinnamic acid<sup>4c</sup>. Third, *p*-toluenesulfinic acid did not react with furylacrylic acid, although reaction does take place with cinnamic acid<sup>4a</sup>. In this connection it is interesting to note that when the reaction was carried out in ethyl alcohol, esterification took place and gave a satisfactory yield of ethyl furylacrylate. Finally, the accepted methods for oxidizing the sulfide probably obtained

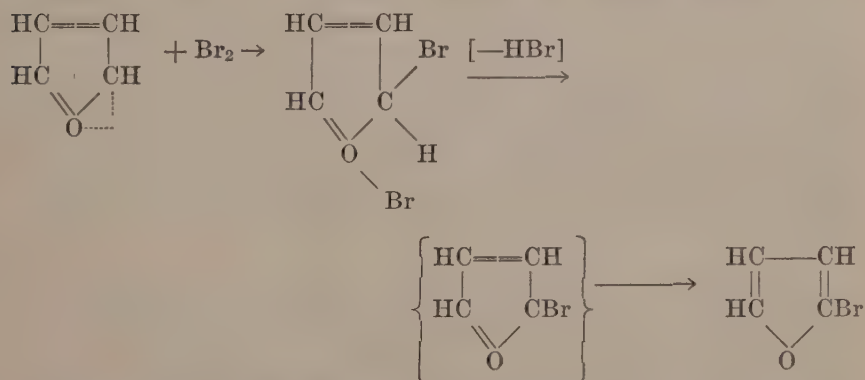
<sup>4</sup>(a) Kohler and Reimer, *Am. Chem. J.*, **31**, 163 (1904); (b) Ruhemann, *J. Chem. Soc.*, **87**, 17, 461 (1905); (c) Arndt, Flemming, Scholz and Löwensohn, *Ber.*, **56**, 1269 (1923); (d) Gilman and King, *J. Am. Chem. Soc.*, **47**, 1136 (1925).

in Reaction (I) to the sulfone obtained in Reaction (II) were not successful. This difficulty is not due entirely to any instability of the furan nucleus to oxidizing agents as is attested by the high recovery of sulfide in some oxidations. In this connection, attention should be directed to the ready polymerization of furfuryl mercaptan<sup>5</sup>.

In connection with preliminary addition to the nuclear oxygen and the ease of *alpha*-substitution, there is a possibility that the analogies between furan and other aromatic types like benzene can be extended to include the phenomenon of oscillating linkages in the related nuclei. We might, for example, have the following in a system like that proposed by Kekulé in his revised formula for benzene:



With Formulas (A) and (B) the active parts would be the oxygen and an *alpha*-carbon atom. In such a representation, with a dynamic system, bromination (as a type) may take place in the following manner:



The bracketed hypothetical bromofuran need not have trivalent oxygen and trivalent carbon, but may have a structure like that represented in Formula (B) which then undergoes a re-allocation of linkages to give the ultimate product. It may be objected that an interpretation of this kind might warrant the expectation of addition to other unsaturated parts of the nucleus, like the ethylenic linkage between carbon atoms 3 and 4. The same objection is patent in the interpretation of preliminary addition in substitution reactions of benzene. Possibly it is just such a labilized system which is necessary to account for the uncommon tendency of furan types to form tetrahalogen *addition* compounds.

<sup>5</sup>Gilman and Hewlett, *Iowa State Coll. J. of Sci.*, **5**, 19 (1930). See, also, Bost, Turner and Norton, *J. Am. Chem. Soc.*, **54**, 1985 (1932), who could not successfully oxidize a furyl sulfide.

## EXPERIMENTAL PART

All reactions were carried out in a three-necked flask of suitable size, equipped with a mercury sealed stirrer, separatory funnel and condenser. A trap<sup>6</sup> was used to reduce atmospheric contamination.

*Furfural and p-Thiocresol*.—A number of experiments were carried out in both benzene and toluene and for varying periods of refluxing up to twenty-four hours. The ratio of *p*-thiocresol to furfural was varied up to 2:1. With 0.1 mole of furfural, about 0.4 g. of piperidine was used as a catalyst. The furfural recovered was as high as 67 per cent. The *p*-thiocresol recovered was as high as 95.6 per cent (in which experiment about 10 per cent was di-*p*-tolylidisulfide).

*Benzoyl Furan and p-Thiocresol*.—A solution of 17.2 g. (0.1 mole) of benzoyl furan and 37.2 g. (0.3 mole) of *p*-thiocresol with 0.5 g. of piperidine in 60 cc. of xylene was refluxed for twenty-five hours. The recovered products were 35 g. of *p*-thiocresol, 1.2 g. of di-*p*-tolylidisulfide (making a total recovery of 97.3 per cent of thiocresol equivalent), and 16 g. or 93 per cent of the benzoyl furan. A small amount of tar was left in the distillation flask. The very high recovery of benzoyl furan is particularly significant from the point of view of the thermal stability of some furan compounds.

*5-Bromofurfural and p-Thiocresol*.—To 8.7 g. (0.05 mole) of 5-bromofurfural (kindly provided by Mr. G. F. Wright) in 50 cc. of xylene was added 18.5 g. (0.15 mole) of *p*-thiocresol and 0.5 g. piperidine. The reaction mixture was refluxed as usual and no apparent reaction took place. At the end of four hours a vigorous reaction set in with the evolution of hydrogen bromide and the deposition of carbon. No 5-bromofurfural was recovered. But the recovery of *p*-thiocresol was 91.8 per cent, of which 43.2 per cent was as di-*p*-tolylidisulfide. The larger than usual quantity of di-*p*-tolylidisulfide may be significant with a relatively labile compound like 5-bromofurfural.

*Furfural and p-Thiocresylmagnesium Iodide*.—After refluxing an ether solution of the components (using an excess of the thiocresylmagnesium iodide) for twelve hours, there was recovered 67.7 per cent of furfural and 95.3 per cent of thiocresol, of which 1.3 per cent was di-*p*-tolylidisulfide. Almost exactly the same quantity of benzaldehyde was recovered in an earlier related study<sup>4d</sup> with *p*-thiocresylmagnesium iodide.

When refluxing was carried out in an ether-xylene solution at 66-71° for twenty hours the tarry mixture yielded no furfural, and 90 per cent of *p*-thiocresol, of which 13.3 per cent was di-*p*-tolylidisulfide.

*Furfural and Benzoyl Furan with p-Toluenesulfinic Acid*.—The furfural in benzene at 60-65° was completely decomposed and di-*p*-tolylidisulfoxide,  $\text{CH}_3\text{C}_6\text{H}_4\text{SO}_2\text{SC}_6\text{H}_4\text{CH}_3$ , was recovered. This disulfoxide undoubtedly arises from the sulfinic acid. The sulfinic acid under milder conditions probably adds to furfural as it does to benzaldehyde<sup>4a</sup>, but this addition compound was not isolated and analyzed.

<sup>6</sup>Gilman and Hewlett, *Rec. trav. chim.*, **48**, 1124 (1929).



Benzoyl furan under corresponding conditions is much more resistant to the action of *p*-toluenesulfinic acid. After refluxing an ether-xylene solution of 15 g. (0.087 mole) of benzoyl furan and 27.1 g. (0.174 mole) of *p*-toluenesulfinic acid at 80° for twenty-four hours, 90 per cent of the benzoyl furan was recovered in addition to di-*p*-tolyldisulfoxide.

*Furfuralacetophenone and p-Thiocresol* (see Reaction (I)).—A vigorous reaction took place between 19.8 g. (0.1 mole) of furfuralacetophenone, 12.4 g. (0.1 mole) of *p*-thiocresol and 0.5 g. of piperidine in 50 g. of benzene. After standing for twenty-four hours the mixture was worked up to give a 90 per cent yield of 1-benzoyl-2-furyl-2-*p*-thiocresylethane (see reaction (I)), which when crystallized from a mixture of 90 per cent petroleum ether (b.p. 40-60°) and 10 per cent benzene melted at 78.5°. This stable, yellow crystalline compound is soluble in chloroform, carbon tetrachloride and hot alcohol.

*Anal.* Calcd. for  $C_{20}H_{18}O_2S$ : S, 9.96. Found: S, 10.6, 10.11.

Several unsuccessful attempts were made to oxidize this sulfide to the corresponding sulfone. With 30 per cent hydrogen peroxide in acetic acid, tars were formed which so far have resisted crystallization. With chromic oxide in acetic acid at 40° about 70 per cent of the sulfide was recovered; and at 100°, decomposition apparently set in. The same phenomena were observed with sodium dichromate in sulfuric acid. With potassium permanganate and acetic acid at moderate temperatures about 50 per cent of the sulfide was recovered.

*Furfuralacetophenone and p-Toluenesulfinic Acid* (see Reaction (II)).—The reaction mixture of 19.8 g. (0.1 mole) of furfuralacetophenone and 15.6 g. (0.1 mole) of *p*-toluenesulfinic acid in 75 cc. of alcohol, filled with needle crystals after twenty-four hours. After standing for four days it was worked up to yield 13.4 g. or 35 per cent of the sulfone: namely, 1-benzoyl-2-furyl-2-*p*-tolylsulfonylethane (see Reaction (II)). On crystallization from a mixture of 80 per cent benzene and 20 per cent petroleum ether it melted at 141°.

*Anal.* Calcd. for  $C_{20}H_{18}O_4S$ : S, 9.06. Found: S, 9.26, 9.20.

*Furfuralacetophenone and p-Thiocresylmagnesium Iodide*.—The general conditions were those used in the related study with benzalacetophenone<sup>4d</sup>. Despite the fact that some sort of reaction occurred, as evidenced by refluxing of the ether and the separation of a red layer, 90 per cent of the thiocresol was recovered. In other experiments using two equivalents of *p*-thiocresylmagnesium iodide and heating on a water bath for one or two days, 92 per cent *p*-thiocresol was recovered. The tar and red gum, probably condensation products of the furfuralacetophenone, have as yet resisted crystallization.

From one experiment with 4 equivalents of *p*-thiocresylmagnesium iodide and refluxing on a water bath for four days, there was recovered 90 per cent of *p*-thiocresol. The oily residue, after several fractional precipitations with petroleum ether (b.p., 40-60°) and benzene yielded a small quantity of the 1-benzoyl-2-furyl-2-*p*-thiocresylethane (see Reaction (I)). It was identified by a mixed melting point determination.

*Furylacrylic Acid and p-Thiocresol.*—The general procedure was that of Arndt<sup>4c</sup>. From the reaction mixture there has been isolated so far some unaltered furylacrylic acid, *p*-thiocresol and an oil which is probably *p*-thiocresyl acetate,  $\text{CH}_3\text{C}_6\text{H}_4\text{SCOHCH}_3$ , for it yields *p*-thiocresol on hydrolysis. It owes its formation to acetylation of *p*-thiocresol by the acetic acid and acetic anhydride used as the medium.

*Ethyl Furylacrylate and p-Thiocresol.*—Equimolecular portions of the reactants in benzene, with piperidine as a catalyst, were allowed to stand for a week. A practically quantitative yield of initial materials was obtained. Similar results were noted in a check experiment carried out at 50-60°. The authors are grateful to Mr. G. F. Wright for the ester.

*Furylacrylic Acid and p-Toluenesulfinic Acid.*—The general procedure followed was that of Kohler and Reimer<sup>4a</sup>. From a first experiment carried out in water, there was a practically quantitative recovery of furylacrylic acid.

From a second experiment with 0.025 mole of reactants in 40 cc. of alcohol, refluxed for fifteen hours, there was obtained 48 per cent of ethyl furylacrylate.

. . . . .

The authors are grateful to Mr. W. F. Schulz for a helpful suggestion.

#### SUMMARY

In connection with mechanisms concerned with nuclear substitution reactions of furan types, a study has been made of conjugated systems which are undoubtedly involved in the addition reactions which precede substitution. The precursory reaction of 1,4-addition to a conjugated system is probably the formation of an oxonium compound by addition to the oxygen which because of a crossed conjugated system or because of oscillating double bonds has an exalted activity. The conjugated system involved in preliminary addition is probably that comprising the two *alpha*-carbon atoms and not a nuclear-lateral system. Completely lateral conjugated systems participate in some characteristic 1,4-additions. However, the exceptions from the related benzene compounds are sufficiently marked to emphasize caution in drawing broad generalizations between the phenyl and furyl series.

# THE STABILIZING EFFECT OF NUCLEAR NITRO GROUPS IN FURAN TYPES

## 5-NITRO-2-FURFURYL CHLORIDE AND 5-NITRO-2-FURFURYL METHYL ETHER

HENRY GILMAN AND ROBERT R. BURTNER

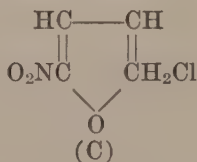
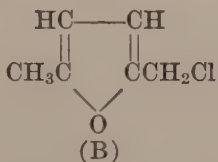
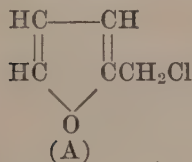
*From the Chemical Laboratory of Iowa State College*

Accepted for publication May 28, 1932

### INTRODUCTION

Current studies on nuclear substitution reactions of furan demonstrate in a striking manner the effect of various substituents on the stability of the furan nucleus. In general, some of these effects might have been predicted from other aromatic types. However, the effect appears to be exaggerated with furan types. For example, no simple aminofuran has as yet been prepared, and the simple *alpha*- or 2-monohalides like chloro-, bromo- and iodo-furan are decidedly less stable than the corresponding analogues in the benzene series. The stability of such types is increased by the introduction of a negative group like carboxyl so that the corresponding amino- (or acetamino) and halogen-furoic acids are of a relatively high order of stability. Such stability is further increased by the introduction of a nitro group as in 4-nitro-5-amino-2-furoate<sup>1</sup>.

Furfuryl chloride (A) is unstable<sup>2</sup>. 5-Methyl-2-furfuryl chloride (B) has recently<sup>3</sup> been shown to be much less stable than furfuryl chloride. However, 5-nitro-2-furfuryl chloride (C) is relatively highly stable.

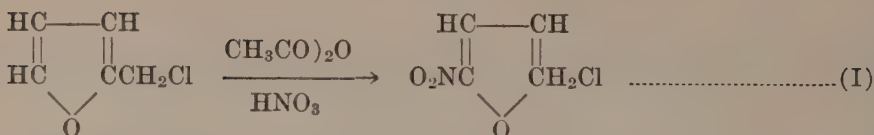


The 5-nitro-2-furfuryl chloride has been synthesized by two different methods. The first of these methods, nitration of furfuryl chloride, is somewhat unusual because of the apparent instability of furfuryl chloride to acids. However, a distinction must be made between halogen acids and other so-called mineral acids; also, it is possible to prepare furfuryl chloride from furfuryl alcohol and hydrogen chloride if due precautions are taken to exclude moisture<sup>2</sup>.

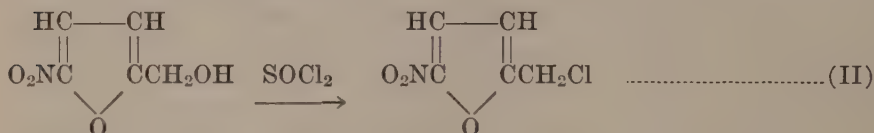
<sup>1</sup>Studies by G. F. Wright indicate that the effect of the negative groups on the amino group in this molecule is such as to endow the amino group with very slightly basic or even acidic properties. Solubility in alkali is very probably due to a transformation like that observed with *o*- and *p*-nitroanilines and derivatives.

<sup>2</sup>v. Braun and Köhler, *Ber.*, **51**, 87 (1918); Gilman and Vernon, *J. Am. Chem. Soc.*, **46**, 2576 (1924); Kirner, *ibid.*, **50**, 1955 (1928).

<sup>3</sup>Reichstein and Zschokke, *Helv. Chim. Acta.*, **15**, 249 (1932).

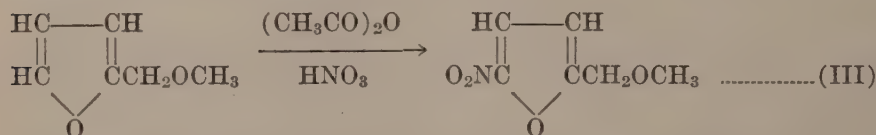


The second method is the action of thionyl chloride on the recently accessible 5-nitro-2-furfuryl alcohol<sup>4</sup>.



The 5-nitro-2-furfuryl chloride was characterized by conversion, by means of silver acetate, to 5-nitro-2-furfuryl acetate<sup>4</sup>. Physiological tests on 5-nitro-2-furfuryl chloride show that the lethal concentration for mice on ten minutes exposure is greater than 7 mg. per liter. Particular care should be exercised in working with 5-nitro-2-furfuryl chloride because of its vesicant action, which is about 1/150 that of mustard gas.

Another illustration of the stabilizing effect of the nitro group is found in 5-nitro-2-furfuryl methyl ether which was synthesized by the nitration of furfuryl methyl ether.



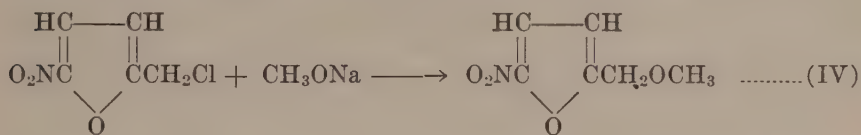
Although furfuryl methyl ether is relatively unstable unless special precautions are observed, the 5-nitro-2-furfuryl methyl ether not only is highly stable without the use of solvents or diluents, but is also resistant to the action of acids. This is strikingly illustrated by experiments with hydriodic acid under conditions for the splitting of ethers. Not only is the heterocyclic ether unit rendered stable to the action of hydriodic acid, but the methoxyl group also appears to be relatively unaffected by this reagent, which is commonly employed in the Zeisel analysis for the splitting of methyl ethers. This unusual stability of a lateral function, induced in large part by the nuclear nitro group, has been observed in another study by Mr. R. V. Young on 5-nitro-2-furfuralmalonic ester wherein the ester groups are uncommonly resistant to hydrolysis.

The nitration of furfuryl chloride and furfuryl methyl ether to give an *alpha*- or 5-nitro substituent is additional evidence that with furan types the tendency to substitute in an *alpha*-position is essentially independent of the group already present. That is, substituents which in the benzene series orient either *ortho* and *para* or *meta*, orient in the furan series to an *alpha* position, if such be available.

<sup>4</sup>Gilman and Wright, *J. Am. Chem. Soc.*, **53**, 1923 (1931).



The position of the nitro group in nitrofurfuryl methyl ether was established by converting 5-nitrofurfuryl chloride to the nitro-furfuryl methyl ether obtained by nitration of furfuryl methyl ether:



This reaction (IV), carried out under anhydrous conditions, is of more than passing interest because of the great sensitivity of *alpha*-nitro compounds to alkali. The stability of 5-nitrofurfuryl methyl ether to hydrogen iodide was ascertained incidental to some attempts to hydrolyze this ether to the known 5-nitrofurfuryl alcohol.

#### EXPERIMENTAL PART

*5-Nitro-2-Furfuryl Chloride.*—This compound was prepared by two different methods.

First, 50.0 g. (0.43 mole) of furfuryl chloride dissolved in 120 cc. of acetic anhydride was added dropwise with stirring at  $-25^\circ$  to the nitrating mixture prepared by the dropwise addition of 130.0 g. (2.0 moles) of fuming nitric acid to 360 cc. of acetic anhydride at  $-5^\circ$ . Following the addition, the mixture was allowed to stir for an additional hour at the indicated temperature and then poured into 1.0 kg. of cracked ice. Neutralization was effected by adding 50 per cent sodium hydroxide until the mixture was faintly acid to methyl orange; then neutralization was completed with a suspension of sodium bicarbonate in water. The mixture was then extracted with ether three times and 100 g. of pyridine added to the ethereal extract. After standing at room temperature for twenty-four hours the ether was distilled off, and approximately three-quarters of the pyridine was removed by distillation under reduced pressure. The residual material was then acidified with dilute hydrochloric acid and extracted with ether. The ethereal extract was dried over anhydrous sodium sulfate and the solvent removed by distillation, fractionating the residue under reduced pressure to obtain 15.0 g. or a 25 per cent yield of a light yellow oil boiling at  $122\text{--}124^\circ/6$  mm.; sp. g.  $_{20}^{20}$  1.429;  $n_D^{20}$  1.5688.

*Anal.* Calcd. for  $\text{C}_5\text{H}_4\text{O}_3\text{NCl}$ : Cl, 21.93. Found: Cl, 21.59.

Second, 59.0 (0.5 mole) of thionyl chloride was added dropwise with stirring at  $0^\circ$  to 71.5 g. (0.5 mole) of 5-nitro-2-furfuryl alcohol<sup>4</sup>. Following the addition of the thionyl chloride the mixture was allowed to stir for two hours longer at  $0^\circ$ , at the end of which period the temperature was slowly raised to  $60^\circ$  and maintained at this point for four hours. The resulting solution was then washed several times with water, taken up with 75 cc. of ether and dried over anhydrous sodium sulfate. The solvent was distilled off and the residue fractionated under reduced pressure, collecting the fraction boiling at  $125\text{--}129^\circ/7$  mm. The yield was 34.0 g. or 41.6 per cent. A small amount of the nitro alcohol was recovered unchanged, showing that the reaction mixture should have been refluxed longer with a slight excess of thionyl chloride. Without additional refluxing, but with a 50 per cent excess of thionyl chloride the yield was 50.5 per cent.

Despite the superior yields of 5-nitro-2-furfuryl chloride by the action of thionyl chloride on 5-nitrofurfuryl alcohol, the method involving direct nitration of furfuryl chloride has its merits. First, it appears to be less time consuming. Second, the over-all yield starting with furfuryl alcohol is about the same in both processes. Third, the distillation of 5-nitrofurfuryl alcohol may, at times, be troublesome due to decompositions in varying degrees of violence. Over and against such advantages, it should also be observed that occasionally the purification of furfuryl chloride is attended with deep-seated decompositions or condensations, as is sometimes observed with benzyl chloride.

*5-Nitro-2-Furfuryl Acetate*.—5.0 g. (0.03 mole) of nitrofurfuryl chloride and 7.6 g. (0.045 mole) of silver acetate were placed in a small three-necked flask with 75 cc. of absolute ether and refluxed with stirring for a period of four hours. The solution was then filtered and the ether removed under reduced pressure, leaving a small crop of yellow crystals. This material was pressed dry on a porous plate and was found to melt at 45°. A mixed melting point with an authentic sample of 5-nitro-2-furfuryl acetate showed no depression.

*5-Nitro-2-Furfuryl Methyl Ether*.—Fifty-six g. (0.05 mole) of furfuryl methyl ether<sup>5</sup> dissolved in 100 cc. of acetic anhydride was added dropwise with stirring at  $-15^{\circ}$  to the nitrating mixture prepared by dropwise addition of 85.0 g. (1.3 moles) of fuming nitric acid to 287 cc. of acetic anhydride at  $-5^{\circ}$ . Following the addition, the reactants were allowed to stir for an additional hour in the freezing mixture and then poured into 2 kg. of cracked ice. The mixture was treated with 50 per cent sodium hydroxide until the reaction is faintly acid to methyl orange and the neutralization then completed with a suspension of sodium bicarbonate in water. The resulting solution was extracted three times with ether and 125 cc. of pyridine was added to the ethereal extract. After standing for twenty-four hours at room temperature the ether was distilled off and approximately three-quarters of the pyridine removed by fractionation under reduced pressure. The remainder of the pyridine was then removed by acidification with dilute hydrochloric acid and the resulting solution extracted with ether. The ethereal extract was dried over anhydrous sodium sulfate and the ether distilled off. The residual material was then fractionated under reduced pressure collecting the light yellow oil boiling at  $114-117^{\circ}/4$  mm.; sp. g.  $_{20}^{20}$  1.283;  $n_D^{20}$  1.5343. The yield was 42.6 g. or 54.2 per cent.

*Anal.* Calcd. for  $C_6H_7O_4N$ : N, 8.91. Found: N, 9.23.

*Attempted Splitting of 5-Nitro-2-Furfuryl Methyl Ether*.—15.7 g. (0.1 mole) of the nitro furfuryl methyl ether was heated at  $60^{\circ}$  under a reflux condenser with 33.6 g. hydriodic acid (sp. g. 1.68) containing a small amount of red phosphorus, for a period of one and one-half hours. The mixture was then poured into 400 cc. of ice water and neutralized with

---

<sup>5</sup>Pummerer and Gump, *Ber.*, 56, 999 (1923).

sodium bicarbonate solution. The reaction mixture was extracted with ether, washed with a solution of sodium thiosulfate, and dried over anhydrous sodium sulfate. The ether was then distilled off and the residual material fractionated under reduced pressure, collecting 11.5 g. of the nitro furfuryl methyl ether unchanged.

A second attempt at splitting of the ether was made using the same amounts of materials but refluxing the mixture at 135° for two hours. Ten g. of the ether was recovered. Part of the remainder of the ether was probably lost by decomposition during the final distillation.

*5-Nitrofurfuryl Chloride and Sodium Methylate.*—A cooled solution of sodium methylate prepared from 2.3 g. (0.1 atom) of sodium and 32 g. of absolute methyl alcohol was added dropwise and with vigorous stirring to a solution of 16.1 g. (0.1 mole) of 5-nitrofurfuryl chloride in 16 cc. of absolute methyl alcohol contained in a three-necked flask immersed in an ice bath. The rate of addition was such that there was practically no rise in temperature.

Subsequent to the addition of the 5-nitrofurfuryl chloride, the dark brown reaction mixture was stirred for ten minutes at room temperature. It was then poured upon about 200 g. of cracked ice and immediately acidified with 10 per cent sulfuric acid. The mixture was extracted with ether; the extract dried over sodium sulfate; the ether removed by distillation; and the residue fractionated under reduced pressure. The small quantity of distillate was shown to be 5-nitrofurfuryl methyl ether by its boiling point and refractive index.

*Oxidation of 5-Nitrofurfuryl Alcohol.*—In establishing the constitution of 5-nitrofurfuryl alcohol<sup>4</sup> it was oxidized to the known 5-nitrofurfural. Subsequent to that study, Mr. G. F. Wright showed that the nitro alcohol could be oxidized, as might have been expected, to 5-nitro-2-furoic acid. Fourteen and three-tenths g. (0.1 mole) of nitrofurfuryl alcohol, 20 g. of manganese dioxide and 50 cc. of 50 per cent sulfuric acid were mixed in a 125 cc. flask and allowed to stand for forty-eight hours at 40-45°. At the end of this time 5 g. more of manganese dioxide was added and the reaction continued for twelve hours longer. It was then cooled, extracted three times with ether and the ether extract washed three times with saturated sodium bicarbonate solution. This bicarbonate solution was then acidified with hydrochloric acid and the solution was extracted with ether. Evaporation of the ether left 1.1 g. of 5-nitrofuroic acid melting at 168°. Crystallization of this acid from water raised the melting point to 182°. A mixed melting point with authentic 5-nitrofuroic acid showed no depression. The yield of crude acid was 7 per cent.

The original ether extract after washing with sodium bicarbonate was dried with sodium sulfate and distilled under reduced pressure. The fraction boiling at 125-130°/4-5 mm. was found to be 2.9 g. of 5-nitrofurfural as proved by conversion to the oxime and a mixed melting point with 5-nitrofurfural. The yield was 21 per cent.

. . . . .

The authors are grateful to Mr. G. F. Wright and Mr. W. H. Zuschwerdt for assistance.

## SUMMARY

The nitration of furfuryl chloride and of furfuryl methyl ether introduces a nitro group in the 5-position. The resulting compounds are unusually stable. For example, whereas furfuryl chloride is unstable and 5-methyl-2-furfuryl chloride is much more unstable, 5-nitro-2-furfuryl chloride is of a relatively high order of stability. The high stability of 5-nitro-2-furfuryl methyl ether is strikingly illustrated by its resistance to the action of hydrogen iodide, both the nuclear and the lateral ether linkages remaining essentially unaltered.

5-Nitro-2-furfuryl chloride was also prepared by the action of thionyl chloride on 5-nitro-2-furfuryl alcohol. Oxidation of 5-nitro-2-furfuryl alcohol gives a mixture of the corresponding nitro-aldehyde and nitro-acid.

5-Nitro-2-furfuryl chloride has a vesicant action.



## THE SULFUR AND NITROGEN OF WOOL

ELEANOR WINTON AND RACHEL EDGAR<sup>1</sup>

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 17, 1932

Although Chevreul (51) in 1806 found less sulfur in black hair than in red or white hair, no work was reported comparing the total sulfur of different kinds of wool until 1867, when Reich and Ulbricht (35) analyzed eight fleeces for sulfur and raised the question of the dependence of the sulfur content upon such factors as the age, breed, sex and portion of the fleece of the sheep from which the wool was obtained.

Trotman and Bell (46, 48) analyzed three wools, found the variation in sulfur slight, and attributed the lack of agreement among the analyses of other investigators to faulty methods of analysis, the probability that the labile sulfur is not entirely a constituent of the protein molecule, and the presence in wool of two or more proteins of different sulfur contents. Marston's work (31) with Australian wools indicated very slight variation in the proportions of sulfur and of nitrogen. Others reported variation in the proportions of sulfur and of nitrogen in wool from different sheep and related these differences to the color, diameter and portion of the fiber, and to the diet of the sheep.

Colored wools have been shown of lower nitrogen content (17, 3). According to Barritt and King (4, 7) and others (2, 3, 1, 44, 24, 8) fine wools contain a higher percentage of sulfur than coarser medullated wools. Yet Bonsma's work (11) with South African wools, the only investigation reported with sheep in a controlled environment, indicates no such relation; Bonsma concluded that the sulfur content of wool was chiefly an individual characteristic, but that nutrition was an important factor in the incorporation of sulfur into the wool fiber. The analysis of the tips and roots of wool fibers has shown that the distribution of sulfur along the length of the fibre is not always uniform. Barritt and King (5) found the tips contained more sulfur than the portions nearer the root and attributed this lack of uniformity to the increased incorporation of cystine after shearing. Such an increase would need to be great enough to overcome the loss of sulfur reported in the oxidation of the tips in light (52, 33, 25). Bonsma found no significant variation in the sulfur of different portions of wool fibers from sheep fed a controlled diet.

Less extensive investigation has been made of the nitrogen than of the sulfur of wools. Total sulfur has been suggested and the determination of nitrogen has been used (39) as a basis for the proximate analysis of the wool of mixtures containing no other source of these elements. The quantitative determinations of sulfur and nitrogen reported in this article were made to obtain more data as to the accuracy of these proximate analyses

---

<sup>1</sup>The authors wish to thank those who gave the wools and Mrs. Vera Berg who developed the method for cleaning the wool.

for wool. Table 1 is a résumé of values reported in the literature for the total sulfur and nitrogen of wools.

TABLE 1. *Values reported for the total sulfur and nitrogen of wools*

Year	Investigator	Sulfur Percent- age	Nitrogen Percent- age	Description of Wool
1840	Chevreur (13)	1.78, 2.20		
1841	Scherer (40)	2.66	17.71	
1856	Von Bibra (53)	0.81, 0.92		mixed
1863	Grothe (18)	1.9, 2.0, 2.1 2.4, 2.5, 2.7 2.0, 2.3, 2.4 2.4, 2.5, 2.5 3.0, 3.2, 3.4 1.6, 1.8, 1.8		carded carded English English Hardschnucke worsted
1867	Reich and Ulbricht (35)	3.37 2.85 3.41 3.55		Lincoln Lincoln Merino yearling Rambouillet ewe Rambouillet Elektoral Negretti yearling
1869	Märcker and Schulze (30)	3.41 3.57 3.66 3.73 3.43 3.69	16.01 16.08 15.86 15.55 15.73 15.54	Landschafen Landschafen Landschafen Landschafen Rambouillet Rambouillet
1870	Henneberg (20)	3.50, 3.62	16.01	wethers
1878	Schützenberger (41)	3.1	17.7	
1882	Bleunard (10)	3.01	12.63 12.90	Australian wool
1885	Bowman (12)	3.0 2.5 2.3 3.8	19.1 18.1 18.5 17.8	Irish Lincoln Northumberland Southdown
	Mulder (12)	5.4	16.8	
	Hummel (22)	3.66	15.86	German
1889	Knecht and Appleyard (26)	3.34, 3.35		
1895	Mohr (34)	3.68		
1901	Washburn (55)	3.42		
1909	Ruszkowski and Schmidt (39)		14.23 13.96	washed fabric washed yarn
1911	Strunk and Priess (43)	3.56	15.14	
1913	— (18)		14.00	flannel
	Gortner (17)		15.11 16.27	black white
1920	Waentig (54)		16.37	
1922	Trotman (47)	3.74	16.01	yarn
1923	Meunier and Latreille (32)	3.0		

TABLE I—Continued

Year	Investigator	Sulfur Percent- age	Nitrogen Percent- age	Description of Wool
1924	Herzog and Krahn (21) Trotman (45)	3.46	16.60 15.70 15.96 16.94	
1926	Barritt and King (4)	3.76 3.33 3.82 3.24 4.00 3.94 3.10 3.26 3.73 3.75 3.82 3.34 3.76 3.97 3.79 3.92		Australian Merino 100's Blackface (coarse) Blackface (fine) Blackface (kempy) Cape Merino (Kaffrarian) Cape Merino (LeGrange) Lincoln (white) Lincoln (yellow) Merino lamb Peruvian (1924) Peruvian (1925) Ripon fleece (fine) Romney (Monte Video) Welsh mountain P64 Welsh mountain S71
	Farrar and King (15) Trotman and Bell (46)	2.9, 3.2, 3.3 3.0, 3.2, 3.3 3.0, 3.3, 3.4		Blackface Lester hog Lester wether
1927	Barritt (2)	3.35 3.64		crossbred Merino
1928	Küster, Kumpf and Köppel (28) Barritt (3)		15.94 16.57 16.63 16.63 16.74 16.76 16.78 16.79 16.82 16.86 17.01 16.69 16.73  16.71 16.16 16.52 16.61 16.80 16.62 16.71 17.07  16.62 16.71 16.50 16.72 16.63 16.74 16.54 16.60 16.68	Australian Merino 60's C22 Australian Merino 64's A6 Australian Merino 58's A3 Australian Merino 60's E67 Australian Merino 64's F87 Australian Merino 60's A4 Australian Merino 60's F86 Australian Merino 64's F85 Australian Merino 60's E64 Australian Merino 64's E76 Australian Merino lamb No. 2 Australian Merino lamb No. 1  Devon lamb Jacob black Jacob white La Concordia Lincoln Lincoln (white) Lincoln (yellow) Mazemet Monte Video  New Zealand matching 50's New Zealand matching 50's Peruvian merino Ripon fleece (fine) Scotch blackface (fine) Scotch blackface (medium) Welsh mountain S10 Welsh mountain P5 Welsh mountain S71

TABLE I—Continued

Year	Investigator	Sulfur Percent- age	Nitrogen Percent- age	Description of Wool
	Marston (31)	3.523	17.9	Leicester
		3.521	17.6	Lincoln
		3.546	17.7	Merino
		3.560	17.8	Merino
		3.562	17.9	Merino
		3.570	17.9	Merino
		3.581	17.7	Merino
		3.585	17.8	Merino
		3.585	18.0	Merino
		3.580	17.9	Merino lamb
		3.535	17.8	Polwarth
		3.541	17.8	Polwarth
		3.530	17.8	Shropshire
	Trotman, Trotman and Brown (49,50)		15.78	crossbred
			15.88	
			15.90	
			16.01	
			16.16	Botany
1929	Barritt and King (5)	3.66		Albury tip 56's
		3.40		Albury root 56's
		3.72		Albury II
		3.08		Albury hogs, tip 58's
		3.00		Albury hogs, root 58's
		3.84		Australian Barratta, New South Wales 60's
		3.73		Australian Barratta, New South Wales 60's
		3.74		Australian Barratta, New South Wales 64's
		3.56		Australian Dalkeith stud ram, New South Wales 64's
		3.48		Australian Dalkeith stud ram, New South Wales 60's
		3.53		Australian Dalkeith 6-tooth ewe 64's
		3.97		Australian Meribee Co. Ltd. stud ram, New South Wales 60's
		3.74		Australian Meribee Co. Ltd. stud ram, New South Wales 60's
		3.69		Australian Millbrae stud 6-tooth ewe, S. Australian 58's
		3.83		Australian Millbrae stud 6-tooth ewe S. Australian 60's
		3.90		Australian Millbrae aged ewe 56's
		3.88		Australian, Mt. Crawford estate, 3 year ram, 58's
		3.91		Australian, Mt. Crawford estate, 3 year ewe, 60's
		3.46		Australian, Mt. Crawford estate, 2½ year ewe, 64's
		3.82		Australian, Triangle Exp. Farm stud ewe, 4 years, 64's
		3.99		Australian, Trangie Exp. Farm wether, 4 years, 70's



TABLE I—Continued

Year	Investigator	Sulfur Percent- age	Nitrogen Percent- age	Description of Wool
1929	Barritt and King (5)	3.93		Australian, Trangie Exp. Farm stud ram, 2 years, 60's
		3.46		Australian, Trangie Exp. Farm stud ram, 5 years, 64's
		4.11		Australian, Trangie Exp. Farm stud ram, 5 years, 60's
		3.77		Australian, Trangie Exp. Farm stud ram, 4 years, 60's
		3.41		Australian Merino tip, E66, 64's
		3.27		Australian Merino root
		3.62		Australian Merino tip E68, 58's
		3.39		Australian Merino root
		3.68		Badger face, white
		3.93		Badger face, white
		3.47		Badger face, brownish black
		3.67		Cape Merino
		3.60		Jacob's flock A, white
		4.05		Jacob's flock B, white
		3.47		Jacob's flock A, black
		3.74		Jacob's flock B, black
		4.01		La Concordia Lincoln
		3.65		Lincoln tip
		3.58		Lincoln II
		3.53		Lincoln III
		3.59		Lincoln root
		3.86		Mazemet
		3.22		New Zealand Romney Corriedale
		3.24		Ripon fleece (fine) tip
		3.33		Ripon fleece (fine) II
		3.40		Ripon fleece (fine) III
		3.27		Ripon fleece (fine) root
		3.22		Romney Corriedale 50's
		3.94		South African Merino
		4.00		South African Merino
		3.67		South African Merino
		4.00		Welsh mountain
		3.98		Welsh mountain
		4.13		Welsh mountain
		4.08		Welsh mountain
		3.75		Welsh mountain
		3.78		Welsh mountain
		4.01		Welsh mountain, all first clip
1929	Barritt and King (6)	3.66		Australian crossbred
		3.48		Australian Merino
		3.34		Devon lamb
		3.27		fabric
		3.47		New Zealand 50's
	Küster and Irion (27)	3.3	16.1	Cape Merino
		3.67		Crossbred 50's I
		3.46		Crossbred 50's II
		3.47		Crossbred 50's III
		3.54		Devon lamb
	Rimington (36)	3.34		Welsh mountain
		4.08		Crossbred 40's
		3.03		
	Rimington (37)			

TABLE I—Continued

Year	Investigator	Sulfur Percent- age	Nitrogen Percent- age	Description of Wool
1931	Bonsma (11)	3.30		half-bred wethers
		3.27		Irish
		3.14		Kent
		3.32		Lincoln
		3.13		Shropshire
		3.55		Shropshire wethers
		3.57		Southdown ewes
		3.43		Southdown tegs
		3.62		Veldt fleece A tip
		3.44		Veldt fleece A middle
		3.71		Veldt fleece A root
		3.60		Veldt fleece A average
		3.54		Veldt fleece B tip
		3.38		Veldt fleece B middle
		3.71		Veldt fleece B root
		3.56		Veldt fleece B average
		3.66		Veldt fleece C tip
		3.63		Veldt fleece C middle
		3.81		Veldt fleece C root
		3.70		Veldt fleece C average
		3.52		Veldt fleece D tip
		3.48		Veldt fleece D middle
		3.48		Veldt fleece D root
		3.48		Veldt fleece D average
		3.31		Veldt fleece E tip
		3.30		Veldt fleece E middle
		3.35		Veldt fleece E root
		3.32		Veldt fleece E average
	Rimington (38)	2.72, 3.06		Welsh mountain birth-coat
	Sidney (42)	3.02		Corriedale
		3.21		Corriedale
		3.16		Romney
		3.17		Romney

## EXPERIMENTAL

The wools analyzed were from registered sheep and were obtained in the grease, the Leicester wool from Mr. J. H. Bohendrier of Elk River, Minnesota, the Cotswold III from Mr. C. H. Hartman of Mt. Emons, Utah, the Karakul wools from the Karakul Fur Sheep Farm at Fayetteville, New York, the Lincoln wool from Dr. D. T. Knight of Marlette, Michigan, the Blacktop Delaine Merino, Cotswold I, Dorset, Hampshire I, Merino B type, Oxford, Rambouillet, Shropshire I, and Southdown I from Michigan State College, the Tunis wool from Mr. R. E. Owen of Fulton, New York, the Cotswold II from Rita M. Smalley of Staatsburg, New York, the Romney from Mr. Eugene Tribble of Lodi, California, and the Columbian, Corriedale, Hampshire II, Shropshire II, and Southdown II from the United States Department of Agriculture.

The raw wool was boiled for 15 minutes in eighty volumes of 0.5 per cent solution of olive oil soap, rinsed thoroughly, immersed in a fresh volume of 0.5 per cent solution of soap for 15 minutes at 50°C., rinsed, again treated for 15 minutes with the soap solution, rinsed, and dried. The wool fibers were then separated by hand in order to remove stained portions and foreign substances. After this sorting the wool was treated

for 15 minutes with 0.5 per cent solution of soap at 50°C., rinsed thoroughly, and, after a repetition of this treatment, immersed for 15 minutes in eighty volumes of water, rinsed, dried, and extracted continuously with ether for 18 hours in a modified Soxhlet extraction apparatus.

The Benedict-Denis method (9, 14) was used for the estimation of sulfur in samples of from two to five grams of wool dried to constant weight at 105°C. The precipitates of barium sulfate were collected on Gooch crucibles (16) and ignited to constant weight at dull red heat in an electric muffle furnace. Blank determinations were made on the reagents with each set of sulfur determinations.

The Kjeldahl-Gunning-Arnold method (29) was used for the estimation of nitrogen in samples of from one and one-half to three grams of wool dried to constant weight at 105°C. Blank determinations were made on the reagents.

Table 2 shows the percentage of sulfur and nitrogen obtained for the wools.

TABLE 2. *Amount of sulfur and nitrogen obtained for the wools*

Wool	Sulfur percentage	Nitrogen percentage
Blacktop Delaine Merino	3.74, 3.75, 3.77, 3.78	15.92, 15.94
Columbia	3.31, 3.32, 3.35	
Corriedale	3.34, 3.36	
Cotswold I	3.33, 3.35, 3.37	16.21, 16.23
Cotswold II	2.99, 3.00, 3.00, 3.01, 3.02	16.25, 16.27
Cotswold III	2.98, 2.99, 3.00	15.81, 15.83
Dorset	3.51, 3.51, 3.54, 3.55	15.92, 15.97
Hampshire I	3.18, 3.19, 3.20	16.34, 16.35
Hampshire II	3.47, 3.50	
Karakul (black)	3.03, 3.04, 3.07, 3.08	15.84, 15.85
Karakul (mixed black and yellow)	2.82, 2.90	15.89, 15.96
Karakul (root)	2.83, 2.86, 2.88	
Karakul (tip)	2.62, 2.64, 2.74	
Leicester	3.44, 3.45	16.30, 16.31
Lincoln	2.91, 2.92, 2.93	15.92, 16.01
Merino B type	3.61, 3.61, 3.63, 3.65	16.18, 16.21
Oxford	3.31, 3.33, 3.34, 3.36	16.20, 16.25
Rambouillet	3.56, 3.59, 3.60, 3.60	15.99, 16.02, 16.03
Romney	2.97, 3.00, 3.02, 3.04, 3.05	16.17, 16.24
Romney (root)	3.21, 3.22	
Romney (tip)	3.60, 3.64	
Shropshire I	3.49, 3.49, 3.50	16.16, 16.17, 16.20
Shropshire II	3.31, 3.32, 3.37	
Southdown I	3.30, 3.33, 3.34, 3.34	16.33, 16.34
Southdown II	3.52, 3.54, 3.57, 3.57	
Tunis (root)	3.46, 3.48	
Tunis (tip)	3.35, 3.35	

#### SUMMARY

1. Sulfur was determined in sixteen wools by the Benedict-Denis method and nitrogen was determined in thirteen wools by the Kjeldahl-Gunning-Arnold method.

2. Analyses of the tips and roots of three wools showed a non-uniform distribution of sulfur along the length of the fiber.

3. The sulfur and nitrogen contents do not furnish bases for the proximate analysis of the wool of mixtures unless blank determinations are made on the wool.

## LITERATURE CITED

1. BARKER, S. G., AND A. T. KING  
1926. A comparison of measurements of diameters of wool fibres with the micro balance and the projecting microscope, with applications to the determination of density and medulla (hemp) composition. *J. Textile Inst.*, **17**:68-74T.
2. BARRITT, J.  
1927. The relation of the cystine yield to the total sulphur in wool. *J. Soc. Chem. Ind.*, **46**:338-141T.
3. —————  
1928. The nitrogen content of naturel and processed wools. *J. Soc. Chem. Ind.*, **47**:69-72T.
4. ————— AND A. T. KING  
1926. The sulphur content of wool. Part I. Inherent variations according to the type of wool. *J. Textile Inst.*, **17**:386-95T.
5. ————— AND —————  
1929. The sulphur content of wool. Part II. Distribution of sulphur along the fibre, variation with colour, and the effect of exposure to ultra violet light. *J. Textile Inst.*, **20**:151-8T.
6. ————— AND —————  
1929. The sulphur content of wool. Part III. Effects of chemical processing on sulphur content. *J. Textile Inst.*, **20**:159-61T.
7. ————— AND —————  
1931. Note on the sulphur-free nature of medulla in blackface wool. *Biochem. J.*, **25**:1075-6.
8. BEKKER, J. G., AND A. T. KING  
1931. Sulphur distribution in the component structures of wool and porcupine quils. *Biochem. J.*, **25**:1077-80.
9. BENEDICT, S. R.  
1909. The estimation of total sulphur in urine. *J. Biol. Chem.*, **6**:363-71.
10. BLEUNARD  
1882. Recherches sur les matières albuminoides. *Ann. chim. Phys.* (5) **26**: 5-85.
11. BONSMAN, F. N.  
1931. The sulphur content of some South African Wools. *J. Textile Inst.*, **22**:305-13T.
12. BOWMAN, F. H.  
1885. The structure of the wool fibre and its relation to the use of wool for textile purposes. *J. Soc. Dyers Colourists*, **1**:136-46.
13. CHEVREUL  
1840. Recherches chimiques sur la teinture. Sur la composition immédiate de la laine.—Sur la théorie de son désuintage et sur quelques propriétés dérivées de sa composition immédiate qui peuvent avoir de l'influence dans les travaux industriels dont elle est l'objet. *Compt. rend.* **10**:631-40.
14. DENIS, W.  
1910. The determination of total sulphur in urine. *J. Biol. Chem.*, **8**:401-3.
15. FARRAR, H. E., AND P. E. KING  
1926. The action of ammonia on wool. *J. Textile Inst.*, **17**:588-90T.
16. FOLIN, O.  
1905. On sulphate and sulphur determinations. *J. Biol. Chem.*, **1**:131-59.



17. GORTNER, R. A.  
1913. Studies on melanin. V. A comparison of certain nitrogen ratios in black and white wool from the same animal. *J. Am. Chem. Soc.*, **35**: 1262-8.
18. GROTHE, H.  
1863. Beiträge zur Kenntniss der Wolle und ihrer Bestandtheile. *J. prakt. Chem.* (1) **89**:420-8.
19. —————  
1913. Testing of bandage material. *Gummi—Ztg.*, **27**:704; through *C. A. A.* **7**: 1812 (1913).
20. HENNEBERG, J. W. J.  
1870. Neue Beiträge zur Begründung einer rationellen Fütterung der Weidkäufer, pp. 268-270. Göttingen: Deuerlich.
21. HERZOG, R. O., AND E. KRAHN  
1924. Proteinstudien. I. Verhalten bei der Auflösung in Phenolen. *Z. physiol. Chem.*, **134**:190-5.
22. HUMMEL, J. J.  
1885. The dyeing of textile fabrics. London: Cassell and Company.
23. KAYE, M.  
1924. Observations on the behaviour of a substance giving the nitroprusside reaction in skin and in hair. *Biochem. J.*, **18**:1289-93.
24. KING, A. T.  
1927. Some chemical aspects of wool research. *J. Textile Inst.*, **18**:361-8T.
25. —————  
1928. Chemical effects of the natural sulphur in wool on the fading of azo dyestuffs. *J. Soc. Dyers Colourists*, **44**:233-6.
26. KNECHT, E., AND J. R. APPELYARD  
1889. On some chemical properties of wool and allied bodies, and on the behavior of these bodies toward the substantive dyes. *J. Soc. Dyers Colourists*, **5**:75-8.
27. KÜSTER, W., AND W. IRION  
1929. Über die Hydrolyse von Wolle durch Natriumsulfid. *Z. physiol. Chem.*, **184**:225-40.
28. —————, W. KUMPF AND W. KÖPPEL  
1927. Über die Hydrolyse von Wolle durch Natriumsulfid. *Z. physiol. Chem.*, **171**:114-55.
29. MAHIN, E. G., AND R. H. CARR  
1923. Quantitative Agricultural Analysis. New York: McGraw-Hill.
30. MÄRCKER, M., AND E. SCHULZE  
1869. Ueber die Zusammensetzung der rohen Schafwolle. *J. prakt. Chem.*, **108**:193-207.
31. MARSTON, H. R.  
1928. The chemical composition of wool with especial reference to the protein of wool fibre (keratin). Australia Council Sci. Ind. Research Bull. No. 38.
32. MEUNIER, L., AND H. LATREILLE  
1923. Chlorage industriel de la laine. *Chimie & industrie*, **10**:636-42.
33. ————— AND G. REY  
1926. Photochimie—action des rayons ultraviolets sur la laine. *Compt. rend.*, **183**:596-8.

34. MOHR, P.  
1895. Ueber den Schwefelgehalt verschiedener Keratinsubstanzen. *Z. physiol. Chem.*, **20**:403-6.
35. REICH, A., AND R. ULBRICHT  
1867. Chemische Untersuchung der Rohwolle mit besonderer Rücksicht auf Race und Fütterungsweise der Thiere, denen sie entnommen. *Ann. Landwirtsch.*, **49**:122-39.
36. RIMINGTON, C.  
1929. The relation between cystine yield and total sulphur in wool. *Biochem. J.* **23**:41-6.
37. —————  
1929. The relation between cystine yield and total sulphur in various animal hairs. *Biochem. J.* **23**: 726-9.
38. —————  
1931. The relation between cystine yield and total sulphur in kemp and outer-coat animal fibres. *Biochem. J.*, **25**:71-3.
39. RUSZKOWSKI, B., AND E. SCHMIDT  
1909. Zur quantitativen Bestimmung von Wolle neben Baumwolle. *Chem. Ztg.*, **33**:949-50.
40. SCHERER, J.  
1841. Chemisch-physiologische Untersuchungen. *Ann.*, **40**:1-64.
41. SCHÜTZENBERGER, P.  
1878. Sur la constitution de la laine et de quelques produits similaires. *Comp. rend.*, **86**:767-70.
42. SIDEY, D. J.  
1931. The sulphur content of some New Zealand wools. *J. Textile Inst.*, **22**:370-3T.
43. STRUNK, H., AND H. PRIESS  
1911. Zur Frage des sulfitarig gebundenen Schwefels in der Wolle. *Z. physiol. Chem.*, **76**:136-44.
44. TAKEDA, T.  
1926. The cystine content of keratins. *Bul. Sci. Fakultato Terkultura*, **2**:262-72; through *C. A.* **22**:2967 (1928).
45. TROTMAN, E. R.  
1924. The relation between the nitrogen of wool and its affinity for acid and basic dyes. *Soc. Dyers Colourists*, **40**:77-9.
46. ————— AND H. S. BELL  
1926. The sulphur content of wool. *J. Soc. Chem. Ind.*, **45**:10-2T.
47. TROTMAN, S. R.  
1922. The chlorination of wool. *J. Soc. Chem. Ind.*, **41**:219-24T.
48. ————— AND H. S. BELL  
1928. The action of chlorine and hypochlorous acid on wool. *J. Soc. Chem. Ind.*, **44**:1116R.
49. —————, E. R. TROTMAN AND J. BROWN  
1928. The action of chlorine and hypochlorous acid on wool. *J. Soc. Chem. Ind.*, **47**:4-8T.
50. ————— AND —————  
1928. The action of formaldehyde on wool. *J. Dyers Colourists*, **44**:49-52.

51. VAUQUELIN, CHEVREUL AND CABALLE  
1806 D'un mémoire sur les cheveux. *Ann. chim. phys.* (1) **58**:41-53.
52. VON BERGEN, W.  
1925. Einfluss des Sonnenlichtes auf Wolle. *Melliand Textilber.* **6**:745-51.
53. VON BIBRA, F.  
1856. Ueber Haare und die Hornsubstanz. *Ann.*, **96**:289-302.
54. WAENTIG, P.  
1920. Zur Bestimmung des Wollgehaltes von Halbwollgarnen und Halbwollgeweben. *Text. Forsch.*, **2**:49-51; throu *Centr. II*, 148 (1920).
55. WASHBURN, C. E.  
1901 The action of caustic soda on wool. *J. Soc. Dyers Colourists*, **17**: 261-4.





## CONTENTS

### Abstracts of Doctoral Theses

Some of the Relationships Among the Organisms in Butter Cultures. MERLE PORTER BAKER.....	409
I. An X-Ray Investigation of the Iron-Copper System. II. A Study of the Corrosion of Galvanized Sheet Iron. JAMES HAL CARTER .....	413
Studies on the Sterilization of Solutions of Glucose and Sucrose. WENDELL BURNHAM COOK.....	417
The Preparation of Per Acids and Their Salts. ROBERT ROY COONS .....	419
Study on the Utilization of Xylose. HSI CH'OU FANG.....	423
Abnormal Reactions of Organometallic Compounds. STANTON A. HARRIS.....	425
The Design of a Plant for the Production of Insulation Board from Agricultural Wastes and Cost Data on This Process. CHARLES EARL HARTFORD.....	429
The Physiological Action of Cystinyl Peptides and Guanidine Derivatives. H. JAMES HARWOOD.....	431
The Effects of Molybdenum and Chromium on the Malleabilization White Cast Iron. EVERETTE LEE HENDERSON.....	435
Furfural and Some of Its Derivatives. AMIOT P. HEWLETT.....	439
A Study of Some of the Lactobacilli. LINCOLN SPENCER HYDE.....	447
Studies in Vitamin A Technic. MARGARET HOUSE IRWIN.....	451
The Volatile Acids Formed from Citric and Lactic Acids by Streptococcus citrovorus and Streptococcus paracitrovorus. MICHAEL B. MICHAELIAN.....	455
The Fermentation of Levulose by Some Bacteria of the Genus Aerobacillus. ROGER PATRICK.....	457
Some Dietary Factors Affecting Lactation in the Albino Rat. LOUISE JENISON PEET.....	463
Preparation of the Lower Chlorides of Silicon. JOSEPH BRADLEY QUIG .....	467
The Development of Synthetic Lumber from Cornstalks. ROGER W. RICHARDSON .....	469
Production of Yeast Growth Stimulants by Molds on Various Media. H. H. SCHOPMEYER.....	471
The Solubility of Rock Phosphate as Influenced by Sulfur and Gypsum. WINFIELD SCOTT.....	473
Band Spectra Produced by Certain Explosion Mixtures. HARLEY A. WILHELM.....	475

---

Note:—Complete copies of these theses can be consulted at the Library, Iowa State College, Ames, Iowa.



# SOME OF THE RELATIONSHIPS AMONG THE ORGANISMS IN BUTTER CULTURES<sup>1</sup>

MERLE PORTER BAKER

*From the Department of Dairy Industry, Iowa State College*

Accepted for publication June 15, 1932

Studies on the bacteriology of butter cultures were made from the standpoint of relationships among the types of organisms present. The work reported is divided into two parts. Part I is a study of the numbers of the two types of organisms present in butter cultures under various conditions. It involves (a), attempts to find a method for determining the numbers of each of the two types of organisms present and (b), studies on the variations occurring in the growth relationships of the organisms in butter cultures under various conditions, including those existing when new cultures are being developed. Part II involves studies on the effect of the addition of calcium carbonate to milk on the keeping qualities of butter cultures made from it.

## PART I. STUDIES ON THE NUMBERS OF EACH OF THE TWO TYPES OF ORGANISMS PRESENT IN BUTTER CULTURES UNDER VARIOUS CONDITIONS

In studying the numbers of each of the two types of organisms present in butter cultures under various conditions several methods for making differential counts were tried. Attempts to distinguish between the two types by the appearance of colonies on agar to which indicator had been added were not successful. The small amounts of acid produced by the colonies diffused out through the medium so that on plates where the number of colonies was large the color change extended over the entire area. On plates where the number of colonies was small they usually all appeared to have produced acid and were assumed to be *Streptococcus lactis*. *S. lactis* is normally present in butter cultures in larger numbers than are the associated organisms and in amounts of butter culture small enough to produce only a few colonies the associated organisms were probably diluted out.

Attempts to determine the numbers of each of the two types of organisms in butter culture by inoculating varying small amounts of butter cultures into litmus milk and using the aroma produced as an index to the presence of the associated organisms and coagulation of the casein or reduction of the litmus as an index to the presence of *S. lactis* were also unsuccessful. The detection of the aroma was difficult in many of the trials and the results secured were not conclusive. The difficulty, in part, was due to the small amount of aroma materials involved and also to the heated odor caused by sterilizing the milk.

The method finally used to determine the approximate numbers of each of the two types of organisms present in butter cultures was to inocu-

---

<sup>1</sup>Original Thesis submitted June, 1931.

late varying small amounts of butter culture into series of flasks containing sterile skimmed milk and, after incubation at 21°C., determine the amount of volatile acidity produced as an index to the presence of the associated organisms and observe coagulation as an index to the presence of *S. lactis*. A comparison of the numbers of each of the two types of organisms in butter cultures of different qualities showed, that in general there were higher numbers of the associated organisms in satisfactory than in unsatisfactory butter cultures, but also, that there were wide variations in the numbers as well as the ratios of the two types of organisms in satisfactory and also in unsatisfactory butter cultures.

Studies were made in which the numbers of each of the two types of organisms added to combinations were varied in order to determine whether adding the associated organisms in the larger numbers and thus giving them a chance to grow more rapidly was a factor in developing satisfactory butter cultures. The results secured showed that adding the associated organisms in large numbers as compared to the numbers of *S. lactis* added tended to increase the amount and also the rate of volatile acid production. However, results secured with this procedure were not constant and showed that under some conditions the associated organisms grow well in combinations even when added in small numbers at the time of preparing the mixtures.

Attempts made to develop butter cultures from combinations which in the original mixtures produced volatile acidities comparable to those produced by satisfactory butter cultures resulted in failures to secure a desirable flavor and aroma.

This indicates that the ability of combinations of associated organisms and *S. lactis* to produce high volatile acidities is not a satisfactory basis on which to select them for use in butter cultures.

In order to determine whether or not some of the difficulty encountered in securing satisfactory butter cultures from certain combinations of associated organisms and *S. lactis* might be avoided by using freshly isolated cultures, experiments comparing freshly isolated and old cultures of *S. lactis* were carried out. The results secured indicated that freshly isolated cultures were no better than old cultures for developing butter cultures. Freshly isolated cultures of associated organisms were not tried because of the time required for their isolation and identification.

## PART II. THE EFFECT OF THE ADDITION OF CALCIUM CARBONATE TO MILK ON THE KEEPING QUALITIES OF BUTTER CULTURES MADE FROM IT

The studies on the effect of the addition of calcium carbonate to milk on the butter cultures made from it included, (1) the effect on the keeping qualities of butter cultures held at room temperature, (2) the relationship between the temperature of holding and the effect of the addition of calcium carbonate on the keeping qualities of butter cultures and (3) the keeping qualities of butter cultures held for long periods of time at room temperature.

There was a small increase in the keeping qualities of butter cultures held at room temperature (25°-32°C.) in milk to which calcium carbonate had been added. The advantage due to the presence of calcium carbonate was greater in the butter cultures held for the longer periods than in the butter cultures held for the shorter periods. In the short holding periods



(5 days or less) the advantage due to the presence of calcium carbonate was only slight. There was no correlation between the effect of the addition of calcium carbonate and the individual butter cultures used.

When comparisons were made of 21°C. and 37°C. as holding temperatures the results showed a considerable increase in the keeping qualities of the butter cultures held at 21°C. in milk with added calcium carbonate but no increase at all in the keeping qualities of the butter cultures held at 37°C. The disadvantages incident to the higher holding temperature were greater than the advantages because of the presence of calcium carbonate. Variations in the keeping qualities secured in the different runs, however, indicated that factors other than the temperature of holding were also important.

Only one of 28 trials in which butter cultures were held in milk with added calcium carbonate at room temperature for long periods of time (154 to 272 days) yielded a satisfactory butter culture when transferred back to pasteurized milk. Microscopic examinations of the material held in the trials usually showed gram positive rods which presumably had survived the pasteurization incident to the preparation of the milk for inoculation. Examinations for the numbers of each the associated organisms and *S. lactis* were made on the material held in four of the trials. These showed wide variations in the proportions of the numbers of the two types of organisms and indicated that some factor or factors other than the loss of one of the types were probably responsible in part at least for the failure of the material to again produce satisfactory butter cultures.



# I. AN X-RAY INVESTIGATION OF THE IRON-COPPER SYSTEM<sup>1</sup>

JAMES HAL CARTER

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

It is quite generally accepted that an immiscibility gap exists in the liquid state of the iron-copper system. Ruer (4) and Benedicks (1) propose that the liquid immiscibility gap intersects the liquidus curve of the phase diagram. On the other hand, Muller (3) claims that there is a lower critical point for the immiscibility gap at about 1500°C. Ruer claims that Muller's results are due to impurities.

The above observations were made by means of thermal and microscopic examinations. The literature contains no report of an x-ray examination of this system.

## EXPERIMENTAL

A series of pure iron-copper alloys, covering the entire range of compositions, were prepared by melting Armco iron and pure copper in a magnesia crucible in connection with an Ajax induction furnace. Nitrogen was passed over the charge during melting to prevent oxidation. The alloys were cooled in air and the condition of the iron and copper in the solidified alloys represents the condition which existed at the time of solidification.

The alloys were examined by application of the Hull-Debye-Sherrer method of x-ray analysis. The alloys were heated at 1700°F. for twelve hours and x-ray photograms made of the annealed samples as well as the original samples. Photomicrographs were made of the samples before and after annealing.

## RESULTS AND CONCLUSIONS

Only iron lines appear in the x-ray photogram of alloys containing up to 13.02 per cent copper. The photograms of alloys containing from 13.02 per cent copper up to and including 82.31 per cent copper show the superimposed x-ray spectra of iron and copper. The alloys containing 83.39 per cent copper at 100 per cent copper show only copper lines.

Heat treatment at 1700°F. for twelve hours produced no change in the photograms, but the photomicrographs of the samples indicate that a change in structure has started. Evidently the time of annealing was insufficient.

The results obtained are in fair agreement with those of Ruer, 23.8 per cent and 85 per cent copper, respectively, for the high copper side of the diagram, but vary for the low copper side. The value obtained for the low copper side agrees well with the result of Benedicks (1).

Future work will be carried out to determine the effect of annealing on the miscibility limits as obtained above. By quenching from high temperatures, it is hoped that further information may be obtained concerning the miscibility gap at temperatures above the melting points of the alloys.

---

<sup>1</sup>Original Thesis submitted December, 1931.

## II. A STUDY OF THE CORROSION OF GALVANIZED SHEET IRON

Localized, or point, corrosion occurs quite generally on galvanized sheet iron. This type of corrosion is more serious than general, or uniform, corrosion because the attack is concentrated on a small area and penetration of the metal results in a very short time. Evans (2) has explained this type of corrosion by his "differential aeration theory."

The present study was undertaken with the view of isolating a certain range of gas compositions most favorable to the progress of localized corrosion.

## EXPERIMENTAL

Samples of 18 gauge galvanized Armco sheet iron, submerged in conductivity water, were subjected to the corrosive action of various mixtures of oxygen, carbon dioxide and nitrogen gases. The compositions of the different gaseous mixtures used are given in table 1.

TABLE 1

Mixture number	P'e't'g. O <sub>2</sub>	P'e't'g. O <sub>2</sub>	Mixture number	P'e't'g. O <sub>2</sub>	P'e't'g. CO <sub>2</sub>
1	5	0.00	11	5	0.06
2	10	0.00	12	10	0.06
3	15	0.00	13	15	0.06
4	20	0.00	14	20	0.06
5	25	0.00	15	25	0.06
6	5	0.03	16	5	0.10
7	10	0.03	17	10	0.10
8	15	0.03	18	15	0.10
9	20	0.03	19	20	0.10
10	25	0.03	20	25	0.10

The samples were perforated at five points on each side by means of a small power drill. The perforations just penetrated the zinc coating.

Three sample cans, each containing sixteen corrosion samples, were fed by each gaseous mixture. The sample cans were also made of the 18 gauge material. The samples were arranged circularly within the can, eight samples at the top and eight at the bottom. The gaseous mixture was introduced at a level between the two layers of samples. The plan of the set-up was that the water in the upper half of the can should be saturated with the gas and a concentration gradient exist in the lower half of the can. As will be pointed out later, this condition did not exist.

The samples were kept at constant temperature,  $26^{\circ}\text{C.} \pm 2^{\circ}$ , by placing the sample cans in a thermostat tank.

The rate at which the gas bubbled through the sample can was about one bubble per second, as measured by a small glass tip through which the gas entered.

The experiment extended from March 1, 1931, to July 24, 1931.



## OBSERVATIONS AND CORRELATIONS

The total number of points on each sample which were attacked and the degree of corrosion were noted. The pH values of solution from the top and bottom portions of forty cans were determined colorimetrically. The dissolved solids of samples of water from twenty cans were determined. The results of these observations are given in tabular form.

Photographs of representative samples corresponding to each gaseous mixture are given in the complete thesis.

The total number of points attacked were summed up for all cans having the same gas composition. The results are given in table 2.

TABLE 2

Pc't'g. CO <sub>2</sub>	5 per cent O <sub>2</sub>	10 per cent O <sub>2</sub>	15 per cent O <sub>2</sub>	20 per cent O <sub>2</sub>	25 per cent O <sub>2</sub>
0.00	282	224	151	136	240
0.03	161	268	111	38	91
0.06	71	82	95	64	227
0.10	100	157	117	227	189

For any constant carbon dioxide content it is found that the corrosion passes through a minimum as the oxygen content increases. Likewise, when the oxygen content is constant and the carbon dioxide is increased the corrosion passes through a minimum. The minimum corrosion occurred at 20 per cent oxygen and 0.03 per cent carbon dioxide.

It is the opinion of the writer that the data obtained on pH values and dissolved solids are insufficient to warrant any conclusions. No correlation could be detected for the data at hand.

The top samples were decidedly more corroded than the bottom samples. Evidently the gases diffused to the top and there was not enough stirring action, nor enough gas, to maintain as high concentration in the lower part of the can as in the upper part. Thus the plan for a concentration gradient in the lower part of the can failed.

Wherever corrosion had proceeded to the extent of formation of a tubercle, it was found that the greatest amount of corrosion had occurred at a point immediately below the original perforation. On the basis of the differential aeration theory, this phenomenon may be explained as follows: corrosion products form about the perforated point on the corrosion sample and stream downward. In the presence of relatively high oxygen concentration, the Fe(OH)<sub>2</sub> formed is oxidized to Fe(OH)<sub>3</sub>. The less soluble Fe(OH)<sub>3</sub> forms a film over the metal surface which is highly resistant to the diffusion of oxygen and thereby causes a region of low oxygen concentration beneath the film. This unaerated portion is anodic to the surrounding aerated portion and electrochemical action results. Iron goes into solution at the anode as Fe(OH)<sub>2</sub> and is deposited as Fe(OH)<sub>3</sub> at the cathodic area. This deposition of Fe(OH)<sub>3</sub> results in the formation of a membranous mantle around the anode. When once the walls of the mantle have formed around the anodic point they will protect it from oxygen, and thus the anodic attack will persist indefinitely.

## LITERATURE REFERENCES

1. BENEDICKS, C.  
1928. Beziehung zwischen Liquiduskurve und flüssiger Mischungslücke. Z. Physik. Chem., **131**, 285.
2. EVANS, U. R.  
1926. Corrosion of metals. Arnold and Company, London, p. 86-97.
3. MULLER, A.  
1927. Über die Mischungslücke in flüssigen Eisen-Kupfer-Legierungen. Z. Anorg. Allgem. Chem., **162**, 231.
4. RUER, R.  
1927. Über die Mischungslücke in flüssigen Eisen-Kupferlegierungen. Z. Anorg. Allgem. Chem., **164**, 366.

# STUDIES ON THE STERILIZATION OF SOLUTIONS OF GLUCOSE AND SUCROSE<sup>1</sup>

WENDELL BURNHAM COOK

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

Glucose, also known as dextrose, corn sugar and cerelese, is being economically produced at the present time by the hydrolysis of corn starch. Upon the modification of the food law regulations in January, 1931, this sugar may now be used in food products without declaring the fact on the label. A promising field thus opened to the producers of glucose is that of the carbonated beverage industry. One of the chief problems of this industry is the production of a product that will not spoil after a period of several months storage (3, 2). Since 85 per cent of the spoilage in carbonated beverage is caused by yeast (5), it is important to study the comparative ease of sterilizing solutions of glucose and sucrose.

Seven strains of spore forming yeasts, typical of those causing spoilage in carbonated beverages, were selected for the experimental work. In order to obtain a sufficient amount of spore material to carry out all of the experiments, spores were prepared by growing them on carrot juice calcium sulfate agar in Kolle flasks. After scraping off the spores, they were dried and diluted by grinding with sterile powdered sugar in an agate mortar.

The concentration of sugars studied were in the range that would be practicable for the manufacturer. The highest concentration of glucose that may be obtained at 25°C. is one of 27° Baumé strength (49.5 per cent). Two other concentrations of syrups were studied—24° Baumé (44 per cent sugar) and 20° Baumé (36 per cent sugar).

The effects of the two sugars on yeast spores were studied in syrups and in syrups containing small amounts of citric acid, both at room temperature (28°C.) and at 60°C. One experiment was conducted using vegetative cells.

For the experiments carried out at room temperature, the following procedure was employed: 100 cc. of a sterile syrup was inoculated with 5 cc. of a solution containing 2 per cent glucose and from one to four million yeasts. At definite intervals, 5 cc. portions of syrup were taken out of the flask and placed in 45 cc. of sterile tap water. The number of organisms present were determined by plating out dilutions on Wort agar and incubating at 28°C. for five days.

The apparatus and techniques used in experiments conducted at 60°C. were similar to those employed by Levine, Buchanan and Lease (4) and Hall (1).

In the experiments conducted at room temperature the results showed that at given concentrations (20°, 24°, 27° Baumé), sucrose syrups favor growth considerably better than glucose. An interesting point brought out was the decrease in the number of organisms in glucose solutions during the first few days of the experiment. In 20° Baumé glucose syrups, the count dropped to 62 per cent of the original number inoculated before

---

<sup>1</sup>Original Thesis submitted August, 1931.

growth occurred; 24° Baumé showed the same decrease, and 27° Baumé showed a decrease of 50 per cent of the initial count. The extent of the lag period was longer in glucose than in sucrose. A 27° Baumé glucose solution showed a lag period of five to seven days, while the same density sucrose solution showed a lag period of one day.

Upon the addition of small amounts of citric acid (.04M) to 27° Baumé syrups, the yeasts in both sugar solutions slowly decreased in number. There was no significant difference on the killing spores between the two sugars.

However, when the same experiment was conducted on yeasts from a 48 hour broth culture, glucose containing .02M and .04M citric acid killed the cells more rapidly than the corresponding sucrose solution.

In the experiments conducted at 60°C., the results showed that it required less time to kill 99 per cent of the yeasts in a sucrose solution than in the corresponding glucose solution.

TABLE 1. *Killing time for 99 per cent of yeast in sugar solutions at 60°C.*

Density Baumé	Citric acid concentration	Glucose	Sucrose
		Time in minutes	
24°	0	72	52
	.02M	34	23
	.04M	27	17
	0	106	90
27°	.02M	43	35
	.04M	38	33

An explanation given for the behavior of the sugar solutions was that the glucose syrup, containing almost twice as many molecules as the sucrose syrup, had a considerably higher osmotic pressure than the sucrose syrups. At room temperature this condition prevented growth of the yeasts for a period until the cells became acclimated to the high osmotic pressure. At higher temperatures, the solutions with the higher osmotic pressure exert a protective action on the cells, tending to hold water in the sugar solution, thus preventing the passage of hot water into the cell.

#### LITERATURE CITED

1. HALL, J. R.  
Chemical sterilization with alkalis. Doctoral thesis. Unpublished. Iowa State College Library, Ames, Iowa.
2. LEVINE, MAX  
1923. Deterioration and spoilage of bottled beverages due to yeast, bacteria and molds. *Beverage News*, 11, no. 2:16-18.
3. ———, J. H. BUCHANAN AND C. E. MCKELVEY  
1925. Some causes of contamination and spoilage of syrups. *Beverage Journal*, 61, no. 1:58.
4. ———, ——— AND GRACE LEASE  
1927. Effect of concentration and temperature on the germicidal efficiency of sodium hydroxide. *Iowa State College Jour. Sci.*, 1:379-394.
5. MCKELVEY, C. E.  
1926. Notes on yeasts in carbonated beverages. *Jour. Bact.*, 11:98-99.



# THE PREPARATION OF PER ACIDS AND THEIR SALTS<sup>1</sup>

ROBERT ROY COONS

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

There are a number of acids to which the prefix "per" has been applied, for example, perchloric, periodic and permanganic. In these cases the prefix "per" denotes only that they contain relatively more oxygen than the chloric, the iodic and the manganic acids. Price<sup>2</sup> defines true per acids "as those which are either formed by the action of hydrogen peroxide on ordinary acids, or else give rise to hydrogen peroxide on treatment with dilute sulphuric acid; with concentrated sulphuric acid many of them evolve ozonized oxygen, thus behaving similarly to the metallic peroxides and to hydrogen peroxide itself."

Price<sup>2</sup> groups the elements forming per acids. Leaving out of consideration the elements in the first two short series of the periodic table, it will be observed that the formation of per acids is confined to groups IV, V and VI, and more particularly, with the exception of tin and selenium to the A family of these groups.

Respecting the elements of family B, Tanatar<sup>3</sup> obtained a perstannic acid by the action of 30 per cent hydrogen peroxide on stannic acid which had been precipitated from a solution of stannic chloride by sodium carbonate. Dennis and Brown<sup>4</sup> reported an impure perselenate, analogous to potassium persulphate, prepared by the electrolysis of a saturated solution of potassium selenate containing a little free selenic acid. Neither Dennis and Koller<sup>5</sup> nor Bauer and Wilkinson<sup>6</sup> were able to confirm this result. Alvarez<sup>7</sup> claims to have prepared sodium perarsenate by dissolving disodium arsenate in water and alcohol, cooling to 0°, and then adding sodium peroxide. Aschkenasi<sup>8</sup> reported perarsenates which were prepared by treating arsenic acid with barium peroxide and hydrogen peroxide, the solid being obtained by the evaporation of the solutions with gentle heating and under reduced pressure.

## PURPOSE OF THE INVESTIGATION

Since no element of a B family has been definitely shown to form per acids or salts, the purpose of this work was to apply to arsenic, a typical element of a B family, all of the known methods for the preparation of per acids.

<sup>1</sup>Original Thesis submitted September, 1931.

<sup>2</sup>Price, *Per-acids and their salts*, pp. 1-8 (1912).

<sup>3</sup>Tanatar, *Ber.*, **38**, 1184 (1905).

<sup>4</sup>Dennis and Brown, *Jour. Am. Chem. Soc.*, **23**, 358 (1901).

<sup>5</sup>Dennis and Kohler, *ibid.*, **41**, 949 (1919).

<sup>6</sup>Bauer and Wilkinson, Unpublished Thesis, Library, Iowa State College, Ames, Iowa (1927).

<sup>7</sup>Alvarez, *Chem. News*, **94**, 269 (1906).

<sup>8</sup>Aschkenasi, German Patent, 296796 (1914).

## METHODS

## 1. HYDROGEN PEROXIDE METHOD

Disodium arsenate was dissolved in a 5 per cent solution of hydrogen peroxide, at room temperature, and the resulting solution was evaporated over concentrated sulphuric acid under reduced pressure.

A second preparation was made by treating a solution of disodium arsenate, saturated at 0°, with 30 cc. of 30 per cent hydrogen peroxide. Before being mixed, the two solutions were cooled to 5°C. No precipitate formed. The mixture was evaporated over concentrated sulphuric acid under reduced pressure and a white residue was left which was very hygroscopic.

The residues were analyzed by the Mohr<sup>9</sup> method for the per cent of arsenic, and by the Bunsen<sup>10</sup> method for the oxidizing equivalent. The second residue was analyzed for the percentage of hydrogen peroxide.

No active oxygen was found in the first residue. From the analytical data of the second residue, which contained 8.45 per cent active oxygen, the calculations show that the product approaches the formula  $25\text{Na}_2\text{HAsO}_4 \cdot 32\text{H}_2\text{O}_2 \cdot 16\text{H}_2\text{O}$ , which is probably a mixture.

## (2) BARIUM PEROXIDE METHOD

Small quantities of barium peroxide were added, at ten minute intervals, to a concentrated solution of arsenic acid kept cold with salt and ice. The filtrate from this preparation was evaporated over concentrated sulphuric acid under reduced pressure.

A second concentrated solution of arsenic acid at 8° was treated with barium peroxide. Sodium hydroxide was added slowly to this mixture, and after filtering, the filtrate was evaporated as indicated in the preceding paragraph.

To a cold solution of disodium arsenate barium peroxide was added in excess. A white solid was obtained by evaporating the filtrate over concentrated sulphuric acid under reduced pressure.

The three residues were analyzed by the Bunsen method for the oxidizing equivalent. No active oxygen was found in the first two residues; the third showed 0.47 per cent active oxygen.

## 3. ELECTROLYTIC METHOD

(A) *Disodium Arsenate*

A solution of disodium arsenate, saturated at 0°, which was made the anolyte inside a porous cup, and a solution of arsenic acid, which was made the catholyte around a porous cup inside a liter beaker, were electrolyzed, at a temperature of 0° to 5°, in the Elb's<sup>11</sup> cell for sixteen hours with an anodic current density of one ampere per square centimeter.

With an anodic current density of 0.7 amperes per square centimeter, a second solution of the same salt, saturated at 0°, was electrolyzed for eight hours at a temperature of 0° to 10°. Two platinum wires were used as electrodes<sup>12</sup>.

<sup>9</sup>Scott, Standard methods of chemical analysis, 1, p. 36 (1927).

<sup>10</sup>Foulk, Notes on quantitative analysis, p. 181 (1930).

<sup>11</sup>Elbs, Electrolytic preparations, p. 35 (1903).

<sup>12</sup>Partington, A textbook of inorganic chemistry, p. 518 (1927).

A third solution of the same salt and of the same concentration was electrolyzed for twelve hours, the temperature ranging from  $0^{\circ}$  to  $8^{\circ}$ , with an anodic current density of 1.5 amperes per square centimeter.

Since no precipitate was obtained in either case, the three solutions were analyzed by the Bunsen method for the oxidizing equivalent. No active oxygen was found in the first two preparations, while the third contained 0.0137 g. of active oxygen per 100 cc. of solution.

#### (B) *Disodium Arsenate and Sodium Fluoride*

Approximately 125 cc. of a solution of disodium arsenate, saturated at  $0^{\circ}$  and containing 1 g. of sodium fluoride, was electrolyzed for ten hours, at  $0$ - $12^{\circ}$ , using an anodic current density of 0.7 to 1.8 amperes per square centimeter.

An equal volume of the same disodium arsenate solution containing 2.5 g. of sodium fluoride was electrolyzed for sixteen hours with an anodic current density of two to three amperes per square centimeter, the temperature ranging from  $0^{\circ}$  to  $10^{\circ}$ . (See reference 12 for the apparatus used.)

Both of these preparations were analyzed for the oxidizing equivalent and neither showed any active oxygen.

#### 4. SODIUM PEROXIDE METHOD

To 25 g. of disodium arsenate, dissolved in 1500 g. of distilled water in a four-liter round bottom flask, 1700 cc. of 95 per cent ethyl alcohol were added. Fifty grams of sodium peroxide were added to this mixture in small quantities and at intervals of five to ten minutes to prevent an appreciable increase in the temperature. The contents of the flask were kept at  $-10^{\circ}$  throughout the procedure. About 500 cc. of absolute ethyl alcohol were added and the copious white precipitate, after being filtered out, was dried over phosphorus pentoxide under reduced pressure.

The precipitate was analyzed and the results are shown in table 1:

TABLE 1. *Analysis of  $\text{Na}_2\text{O}_2$  precipitate*

Bunsen method percentage $\text{Na}_2\text{O}_2$	$\text{KMnO}_4$ method percentage $\text{Na}_2\text{O}_2$	Mohr method percentage arsenic	Total sodium percentage	Titration with HCl percentage sodium
23.26	22.74	6.26	21.25	17.48
23.36	22.87 22.84	6.23 6.24	21.34	17.16 17.38
23.10	22.90	6.22		17.53
23.23				
23.24				
21.30				

From the data of this table the calculations show that the product approaches the compound  $2\text{Na}_3\text{AsO}_4 \cdot 7\text{Na}_2\text{O}_2 \cdot 2\text{NaOH} \cdot 7.5\text{H}_2\text{O}$ , but is probably a mixture. No perarsenate was present.

#### 5. FLUORINE METHOD

The apparatus used in generating free fluorine was similar to that employed by Jones<sup>13</sup>. The gas was obtained by the electrolysis of fused potassium bifluoride.

<sup>13</sup>Jones, Jour. Phys. Chem., 33, 801 (1929).

(A) Acid Solution. For three hours gaseous fluorine was passed into a solution of disodium arsenate, saturated at  $0^{\circ}$ , the temperature remaining constant. A small amount of a blue precipitate was formed. The filtrate was placed in an automatic refrigerator until analyzed. The precipitate was dried over concentrated sulphuric acid under reduced pressure, and then over phosphorus pentoxide for three days at room temperature.

The filtrate and the precipitate were analyzed by the Bunsen method for the oxidizing equivalent, and contained neither perarsenate nor active oxygen.

(B) Basic Solution. A solution of normal sodium arsenate, with a slight excess of sodium hydroxide, was saturated at  $0^{\circ}$ . A portion of this filtrate, maintained at zero degrees  $\pm 2^{\circ}$ , was treated with free fluorine for three and one-half hours. A bluish-gray precipitate was obtained.

Analyses of the filtrate by the Mohr method for the oxidizing equivalent showed 0.0142 g. of active oxygen per 100 cc. of solution, and neither ozone nor peroxide were found. The precipitate contained no active oxygen.

#### CONCLUSIONS

(1) Five per cent hydrogen peroxide gives no evidence of the formation of a per salt but the 30 per cent hydrogen peroxide shows the formation of a compound with an active oxygen content of 8.45 per cent. The formula indicated is  $25\text{Na}_2\text{HAsO}_4 \cdot 32\text{H}_2\text{O}_2 \cdot 16\text{H}_2\text{O}$ , which is apparently a mixture.

(2) Barium peroxide forms no perarsenate with either disodium arsenate or arsenic acid.

(3) There is no evidence of a perarsenate being formed by the electrolysis of a solution of disodium arsenate either in the presence or absence of fluorides.

(4) Sodium peroxide precipitates a compound from an alcoholic solution of disodium arsenate whose analysis indicates the compound  $2\text{Na}_3\text{AsO}_4 \cdot 7\text{Na}_2\text{O}_2 \cdot 2\text{NaOH} \cdot 75\text{H}_2\text{O}$ , but this is undoubtedly a mixture since different preparations vary in analyses.

(5) Fluorine gas passed into a solution of trisodium arsenate gives but little evidence of the formation of a per salt.



# STUDY ON THE UTILIZATION OF XYLOSE<sup>1</sup>

HSI CH'OU FANG

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

Xylose occurs widely distributed as a condensation product in all cellulose plant material. Studies on the utilization of plant residues which constitute our greatest agricultural waste must necessarily include the study of this constituent. The investigations reported in this work for the utilization of xylose are limited to the three following phases of the general problem:

- I. Oxidation of xylose to xylonic acid;
- II. Preparation of pyrrole from ammonium xylonate;
- III. Fermentation of xylose to butyl alcohol. The present investigation is limited to the development of an analytical method for the determination of a mixture of butanol, acetone and ethanol.

## EXPERIMENTAL

### I. OXIDATION OF XYLOSE TO XYLONIC ACID

Studies were made on the oxidation of xylose solutions by means of gaseous chlorine using ammonium hydroxide as a neutralizing agent. For every volume of gas, room temperature and barometer reading were recorded and the gas volume corrected to standard conditions. At the end of the reaction, that is, after an equivalent amount of chlorine had been absorbed, the solution was concentrated under reduced pressure until ammonium chloride crystallized. After filtering out ammonium chloride, the process of concentrating was repeated several times until no ammonium chloride crystals were shown when the solution was syrupy.

The thin syrup was stored in the ice box for about three weeks, with frequent stirring. About 30 per cent of ammonium xylonate was obtained. M.P. 120-122°.  $(\alpha)_{D}^{27} = +6.2$

Anal. Calcd., 7.617 per cent N. Found, 7.618 per cent N  
7.618 per cent N

Experiments showed that the yield depended on the pH, but no practical method of controlling this factor was found.

### II. PREPARATION OF PYRROLE FROM AMMONIUM XYLONATE

Dry distillation of ammonium xylonate yielded pyrrole in quantities of about 25 per cent of the theoretical. It seems possible that, with larger quantities which would permit more detailed studies, these yields would be increased. This salt would thus be as practical a source of pyrrole as mucic acid if it could be made available.

---

<sup>1</sup>Original Thesis submitted June, 1931.

When other salts such as ammonium arabonate, ammonium gluconate and ammonium galactonate were dry distilled, they also yielded pyrrole; but the yield was very low.

### III. ANALYSIS OF AQUEOUS SOLUTIONS OF BUTANOL, ETHANOL AND ACETONE

A rapid and accurate method of analysis of the products formed is necessary for the study of any fermentation process. Bogin<sup>2</sup> reports that turbidity determinations can be used for the analysis of ternary mixtures of butanol, ethanol and acetone where one constituent is less soluble than the others. The method used by Reilly and his co-worker<sup>3</sup> is limited to the binary system and requires the chemical determination of one constituent for the analysis of the mixture.

The method as developed in this laboratory depends on (a) the determination of acetone by the standard iodine titration method, (b) the measurement of specific gravity of the ternary solution, (c) the measurement of refraction of the ternary solution, and (d) the determination of butanol and ethanol by means of simultaneous equations (as shown below) involving the additive properties of specific gravity and refractivity measured above. Since the method of analysis was designed for our own particular fermentation mixtures, special attention has been devoted to the ratio of solvents which are about 6 parts of butanol, 3 parts of acetone and 1 part of ethanol.

The following equations are for the calculation:

$$S_{B+A+E} = -0.0014C_B - 0.0015C_A - 0.0017C_E + 1 \quad \text{Equ. 1}$$

$$R_{B+A+E} = 2.80C_B + 1.98C_A + 1.73C_E + 13.26 \quad \text{Equ. 2}$$

where A = Acetone,

B = Butanol,

E = Ethanol,

S = Specific gravity,

C = Concentration of solute in grams per 100 cc. of solution, and

R = Reading of immersion refractometer on original solution.

The accuracy of the method is shown in table 1.

TABLE 1. *Results*

Soln.	Specific gravity	Reading of immersion refractometer	Gms. per 100 cc. of solution				
			Acetone by iodine titration	Butanol calc. actual		Ethanol calc. actual	
1	0.9943	23.22	1.2	2.6	2.4	0.19	0.4
2	0.9914	28.12	1.8	3.83	3.6	0.31	0.6
3	0.9884	32.96	2.4	5.05	4.8	0.50	0.8
4	0.9825	42.78	3.6	7.27	7.2	1.15	1.2

<sup>2</sup>Bogin, Ind. Eng. Chem., 16:380-385 (1924).

<sup>3</sup>Reilly & Ralphy, Sci. Proc. Royal Dublin Soc., 15:597-60 (1919).

# ABNORMAL REACTIONS OF ORGANOMETALLIC COMPOUNDS<sup>1</sup>

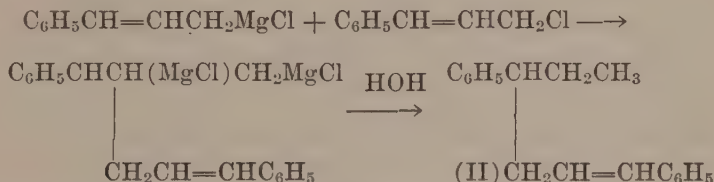
## A. THE ABNORMAL REACTION OF CINNAMYL CHLORIDE WITH MAGNESIUM

STANTON A. HARRIS

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

The object of this work was to find an explanation for the formation of the two hydrocarbons from cinnamyl chloride and magnesium. Rupe and Burgin<sup>2</sup> obtained dicinnamyl ( $\text{C}_6\text{H}_5\text{CH}=\text{CHCH}_2\text{CH}_2\text{CH}=\text{CHC}_6\text{H}_5$  (I)) and a liquid hydrocarbon whose formation they explained by the following reactions:



A new explanation was found in the formation of rearranged products when so-called cinnamylmagnesium chloride was treated with a variety of reagents. The equations illustrating this rearrangement are given on page 426.

Ordinary methods for the preparation of a Grignard reagent gave poor yields with cinnamyl chloride, because of the unusual reactivity of the halogen which causes coupling reactions to take place. Some of the factors which were found to affect the yield were: the purity of the cinnamyl chloride; the amount of surface of magnesium which was available; the rate of addition of the halide; and the amount of ether that was used.

Cinnamyl chloride of high purity, as shown by the absence of tarry residues on distillation, was made in 83 per cent yield by adding a chloroform solution of thionyl chloride. The hydrogen chloride was removed from the reaction mixture by a slight excess of pyridine.

The Grignard reagent was made in 82-87 per cent yield by slowly adding cinnamyl chloride, dissolved in fifteen molecular equivalents of ether, to six atomic equivalents of 30-60 mesh magnesium. The reaction was first started with a small quantity of the cinnamyl chloride solution. Rapid stirring was used throughout the addition of the halide which took 2.25 hours for a two-tenths mole run.

This Grignard reagent acted as if it had the following structure:  $\text{C}_6\text{H}_5\text{CHCH}=\text{CH}_2$ . With carbon dioxide, phenylvinylacetic acid



<sup>1</sup>Original Thesis submitted July, 1931.

<sup>2</sup>Rupe and Bürgin, *Ber.*, 43, 172 (1910).

( $\text{C}_6\text{H}_5\text{CHCH}=\text{CH}_2$ ) was obtained. It was crystallized from petroleum

$$\begin{array}{c} | \\ \text{COOH} \end{array}$$

ether (b.p. 40-60°) at -10° and was found to melt at 23-24°. Its structure was proved; first, by ozonization which gave formaldehyde; and, second, by reduction to  $\alpha$ -phenylbutyric acid. The liquid acid was converted to methylatropic acid ( $\text{C}_6\text{H}_5\text{C}=\text{CHCH}_3$ ) by the action of dilute acid, dilute



base, or by heat alone.

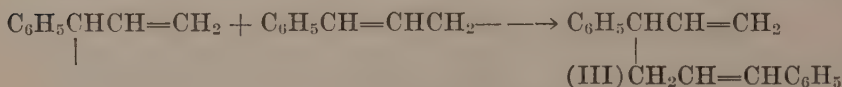
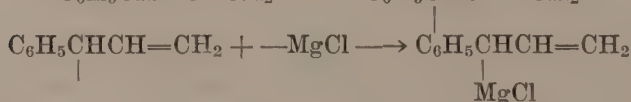
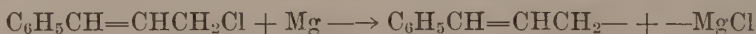
Phenyl isocyanate, with this Grignard reagent, gave phenylvinylacetanilide ( $\text{C}_6\text{H}_5\text{CHCH}=\text{CH}_2$ ) which was found to be identical with the



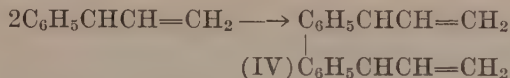
anilide obtained from phenylvinylacetic acid, thionyl chloride and aniline. Ethyl chlorocarbonate gave an ester which yielded methylatropic acid on hydrolysis. The product from gaseous formaldehyde was not identified. On oxidation it yielded only benzoic acid. Similar oxidation of residues from several cinnamylmagnesium chloride runs did not yield any *o*-phthalic or terephthalic acids. This proved that no rearrangement to the ring had taken place, after the kind of rearrangement shown by benzylmagnesium chloride types.

The products obtained from cinnamylmagnesium chloride with oxygen and with ethyl sulfate seemed to support the rearrangement reaction. The attempted catalytic reduction of this Grignard reagent resulted in no addition of hydrogen. The reaction with benzophenone yielded no benzopinacol which showed that this  $\text{RMgX}$  compound did not dissociate appreciably into  $\text{R}-$  and  $-\text{MgX}$ .

The liquid hydrocarbon from cinnamyl chloride and magnesium was carefully separated from dicinnamyl, purified and analyzed. The analysis, ozonization, reduction and comparison of the reduced liquid hydrocarbon with synthetic 1,4-diphenylhexane showed it to have structure (III) instead of structure (II). The following equations were given to explain the formation of the derivatives from cinnamylmagnesium chloride:

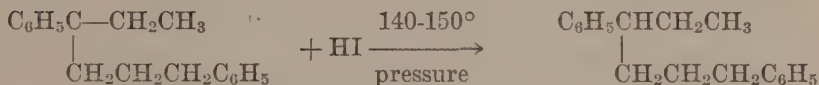
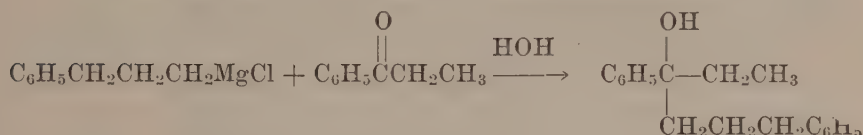
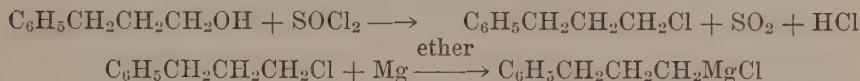






Dicinnamyl (II) was obtained in small yields, but 3,4-diphenylhexadiene-1,5 (IV) was not isolated from the products.

1,4-Diphenylhexane was made by the following reactions:



Ozonization of cinnamyl chloride gave formaldehyde. The molecular refraction showed an exaltation which was over two. This showed the presence of conjugation in the molecule which supports the following structure:  $\text{C}_6\text{H}_5\text{CH}=\text{CHCH}_2\text{Cl}$ . The Grignard reagent acts as if it had the following structure:  $\text{C}_6\text{H}_5\text{CHCH}=\text{CH}_2$ . However, it is possible that



the normal product ( $\text{C}_6\text{H}_5\text{CH}=\text{CHCH}_2\text{MgCl}$ ) is first formed and that rearrangement takes place during the formation of a derivative.

#### B. GRIGNARD REAGENTS FROM HALOGENATED TERTIARY AMINES

Inorganic lead compounds have been used with varying success in cancer therapy. For this reason, it is desirable to obtain water soluble organolead compounds which might have some value for the same purpose. If a Grignard reagent could be made that contained a tertiary amine group in its hydrocarbon radical, it could then be introduced into organolead compounds to give them water solubility.

This work was the continuation of the problem which was initiated by Gilman and Heck<sup>3</sup>. A variety of halogen substituted pyridines were prepared by previously described reactions. 3-Bromopyridine and 3,5-dibromopyridine did not react with activated magnesium-copper alloy in ether solution. They could be made to react at elevated temperatures with or without the presence of ether. No Grignard reagent could be isolated from the tarry mass that was obtained. A color test for the Grignard reagent was obtained if Michler's ketone was added before the reaction went to completion.

<sup>3</sup>Gilman and Heck, *Ber.*, **62**, 1379 (1929).

2-Iodopyridine reacted readily with magnesium in ether solution to give an intensely green solution and an ether insoluble tar. The solution would give no color test, but the entire contents of the reaction flask would give the color test if Michler's ketone was added immediately. No derivatives with carbon dioxide or phenyl isocyanate were obtained. The polymerization reactions were thought to be due to the addition of the Grignard reagent to the carbon-nitrogen double bond.

*o*-Iododimethylaniline and *o*-bromodimethylaniline were found to react very readily with magnesium in ether solution with a small amount of iodine as a catalyst. In 0.05 mole runs the yields were approximately 90 per cent. No derivatives have as yet been made. These results were quite different from those which have been reported for *p*-bromodimethylaniline. Its Grignard reagent is made with extreme difficulty and in small yields.

# THE DESIGN OF A PLANT FOR THE PRODUCTION OF INSULATION BOARD FROM AGRICULTURAL WASTES AND COST DATA ON THIS PROCESS<sup>1</sup>

CHARLES EARL HARTFORD

*From the Department of Chemical Engineering, Iowa State College*

Accepted for publication June 15, 1932

The development of the design of a plant for the production of insulation board from agricultural wastes and the establishment of cost data on this process have resulted from commercial scale work which has been carried on at Dubuque, Iowa, since November, 1929.

The small scale development work which was done prior to and which led up to the commercial scale development which is here reported, was carried on by the United States Bureau of Standards and the Iowa Engineering Experiment Station at Ames, Iowa.

The Dubuque process, from which the following data have been taken, consists of the following steps: the raw material is cut to small pieces, cooked with hot water, washed, put through a series of refining engines, formed into a sheet, pressed, dried and sawed to various sizes.

Field corn, broom corn, pop corn, sweet corn, cornstalks and straw have been tried as raw materials. Field corn cornstalks proved to be the best all-around material. Sweet corn and broom corn cornstalks require a longer cooking time than the field corn cornstalks. Pop corn cornstalks yield a board of lower strength than field corn. Straw gives satisfactory results in every way except that the board is apt to show more coarse particles on the surface.

Four machines, the Williams swing hammer mill shredder, Smalley cutter, Taylor-Stiles rag cutter, and Fox bale breaker and cutter have been tried for dry-cutting the stalks. The power consumed by these machines ranges from 15 kw-hr. per ton of board for the Smalley to 26 for the Williams. The Fox machine, which consumes 16 kw-hr., is the most satisfactory cutter when all factors have been considered.

A Williams swing hammer mill has been used successfully as a shredder for the wet pulp following the dry-cutting and cooking steps. Its power consumption is 12 kw-hr. per ton of board.

The Claflin, Jordan and Bauer machines, which are used to complete the refining of the fibers, consume respectively 57, 165 and 135 kw-hr. per ton of board.

Close control is required in the cooking process. Undercooked pulp gives a board which is light in weight and low in strength, while an overcooked pulp results in a board which is too dense. The correct cooking conditions were found to be 5,000 pounds raw material, 2,200 gallons of water, and a cooking period of one hour and thirty minutes after a temperature of 280°F. has been reached. The temperature is brought to 316°F. and maintained at this point by introducing steam into the pulp.

---

<sup>1</sup>Original Thesis submitted September, 1931.

The rosin-alum method of sizing the pulp is used at Dubuque. Three per cent rosin size is used based on the weight of finished board. The rosin is introduced at a point following the wet shredder, and alum is added just before the pulp is formed into a sheet. Enough alum (about 6 per cent) is used to adjust the pH to 4.5.

The refined and sized pulp is diluted to a consistency of 2.5 per cent with "white water" from the forming machine and press and is then run onto a specially designed forming machine. In this machine a spiral weave reinforced wire carries the pulp over a draining area, then over a suction box which reduces the moisture content of the mat to about 90 per cent, then through a series of five press rolls which reduce the moisture content to about 70 per cent.

The board emerging from the press is cut into 20-foot lengths and fed automatically to the eight decks of a steam heated Coe continuous drier. The drying time is about 168 minutes, and the steam cost is about \$1.00 per thousand square feet of one-half inch thick board.

As the board emerges from the drier it is automatically cut into standard size sheets, and may be stapled or glued to various thicknesses for special purposes.

In locating the factory the most important consideration is nearness of raw material. If the plant is located in a good corn producing area the average haul for the raw material should be about four miles, making the hauling cost \$1.54 per ton of board. This cost increases to \$6.70 for a haul of 225 to 250 miles. Other important considerations in choosing the location are: nearness to market, cost of electric power, water supply, cost of fuel for heating drier.

Cost figures indicate that board can be manufactured for \$18.84 per thousand square feet in a plant designed to produce a board 4 feet wide; for \$15.74 in an 8 foot plant; and for \$14.89 in a 12 foot plant. The 4 foot plant cost is \$215,550; 8 feet, \$292,900; and 12 foot, \$381,750.

By reducing the cooking to a mere soaking in hot water, and mixing in about 15 per cent newsprint, the cost of producing the board in the 12 foot plant is reduced to about \$12 and the cost of the plant to \$352,000. The board produced by this process is light grey in color compared to the tan color of the cooked board. Its strength, weight, moisture resistance and insulation value are about the same as for the cooked pulp board.



# THE PHYSIOLOGICAL ACTION OF CYSTINYL PEPTIDES AND GUANIDINE DERIVATIVES<sup>1</sup>

H. JAMES HARWOOD

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

## I. GUANIDINE DERIVATIVES

The observation that the administration of guanidine hydrochloride resulted in a decrease in blood sugar suggested to a number of workers the possibility that a substance might be discovered which could be used as a substitute for insulin. Of the many compounds studied, one, synthalin (decamethylene diguanidine), because of its very marked hypoglucemic action was recommended for clinical use as a substitute for insulin. Further study of this compound, however, showed it to have an action fundamentally different from that of insulin. Synthalin was also unreliable and in many cases quite toxic. As a result of these findings the search for an insulin substitute has been continued. In the present work a variety of guanidine derivatives and related substances have been prepared for testing as to their effects on blood sugar.

The following compounds were prepared by methods described in the literature: phenylguanidine, diacetoneguanidine, acetyldiacetoneguanidine, formylguanidine, dibenzoylguanidine, anhydrodiacetaylguanidine, dicarboethoxyguanidine,  $\beta$ -carboxylpropionylguanidine, malonylguanidine, thiobarbutic acid, iminimethyluracil, *N*'-allyl-*N*"-guanyl-thiourea, dipiperidyl disulfide, dipiperidyl sulfone, dethylaminomethyl ethyl sulfide, benzimino phenylthioether, diformamidine disulfide hydrochloride and propionomidine hydrochloride.

*p*-Bromophenylguanidine, *p*-carboxylphenylguanidine and benzylguanidine were prepared by the action of a salt of the corresponding aniline derivative on cyanamide. Benzylguanidine, furfurylguanidine sulfate and  $\beta$ -phenylethylguanidine sulfate were prepared by the action of the corresponding amine on methylisothiurea sulfate. Guanidine benzoate was prepared from guanidine - and -benzoic -acid. 1,3-Diphenyl-3-guanidino-propane-1 was obtained by the action of guanidine on benzalacetophenone. Furylacrylylguanidine was prepared by the action of guanidine on ethylfurylacrylate and, similarly, cinnamylguanidine was prepared by the action of guanidine on ethyl cinnamate.  $\gamma$ -Guanidinopropyl *n*-butyl sulfide was prepared by the action of  $\gamma$ -aminopropyl *n*-butyl sulfide on methylisothiurea sulfate. This amine was obtained by treating  $\gamma$ -bromopropylphthalimide with *n*-butyl mercaptan in sodium ethylate solution; the resulting phthalimido compound was then hydrolyzed to the amine. Similarly,  $\gamma$ -guanidinopropyl  $\beta$ -phenylethyl sulfide and  $\gamma$ -guanidinopropyl cyclohexyl sulfide were obtained starting with  $\beta$ -phenylethyl mercaptan and cyclohexyl mercaptan, respectively. *n*-Butylthiobarbituric acid was prepared from *n*-butylmalonic ester and thiourea.

---

<sup>1</sup>Original Thesis submitted September, 1931.

The physiological tests were made by intravenous injections into starved rabbits followed by periodical blood sugar determinations<sup>2</sup>.

Compounds which had no effect on the blood sugar were: *p*-carboxyl phenylguanidine, diacetoneguanidine, furylacrylylguanidine hydrochloride, cinnamylguanidine hydrochloride, formylguanidine, dibenzoylguanidine, anhydro-diacetylguanidine,  $\gamma$ -guanidinopropyl *n*-butyl sulfide sulfate, *n*-butylthiobarbituric acid, iminomethyluracil, *N'*-allyl-*N''*-guanyl-thiourea, dipiperidyl disulfide, dipiperidyl sulfone and diethylaminomethyl ethyl sulfide.

Compounds which raised the blood sugar were: 1, 3-diphenyl-3-guanidino-propanone-1 and hydrochloride,  $\beta$ -carboxylpropionylguanidine,  $\gamma$ -guanidinopropyl cyclohexyl sulfide sulfate, malonylguanidine, thiobarbituric acid, benzimino phenylthioether, hydrochloride, diformidine disulfide hydrochloride and propionamidine hydrochloride.

Compounds which lowered the blood sugar were: phenylguanidine, *p*-bromophenylguanidine nitrate, benzylguanidine nitrate, furfurylguanidine sulfate,  $\beta$ -phenylethylguanidine sulfate, guanidine benzoate, acetyl-diacetoneguanidine, dicarbethoxyguanidine, and  $\gamma$ -guanidino-propyl  $\beta$ -phenylethyl sulfide sulfate.

While the above compounds showed hypoglucemic action, the action in no case approached that of insulin or synthalin and in most cases was accompanied by toxic effects. None of the compounds studied could be recommended as a substitute for insulin.

#### II. CYSTINYL PEPTIDES

The preparation of insulin as a crystalline product with definite physical properties was followed by a large amount of work on the chemical behavior of the hormone. These studies showed insulin to possess the properties of a protein or polypeptide containing the amino acids cystine, tyrosine, arginine, histidine, leucine and perhaps lysine. Whether the activity of insulin is due to the molecule as a whole or to some group within the molecule has not been determined. It appears, however, that the activity bears some relation to the sulfur content of the molecule and that it is also in some way dependent upon the presence of a free amino or imino and carboxyl group.

In the present work a number of polypeptides were prepared consisting of cystine in combination with other amino acids found in insulin. The hope was to find some grouping which would display the hypoglucemic action of insulin. Cystine was included in all of these combinations since it contains the very reactive disulfide linkage which, it has been suggested, might be concerned as a catalyst in the metabolic oxidation of sugar.

Dicarbethoxytyrosylcystine diethylester was prepared by the action of dicarbethoxytyrosyl chloride on cystine diethylester. The latter compounds were prepared by methods described in the literature. Dicarbethoxycystinyltyrosine ethylester was prepared by the action of dicarbethoxycystinyl chloride on tyrosine ethylester. Dicarbethoxycystinyl chloride was obtained by the action of ethyl chlorocarbonate on an alkaline solution of cystine followed by treatment of the free acid with phosphorus pentachloride. Dicarbethoxycystinyltyrosine was prepared by hydrolysis of

<sup>2</sup>The compounds were tested in the laboratories of Parke-Davis and Company.

the ester and also by the action of dicarbethoxycystinyl chloride on an alkaline solution of tyrosine. Dicarbethoxycystinylhistidine methylester was prepared by the action of dicarbethoxycystinyl chloride on histidine methylester. Dicarbethoxycystinylarginine was prepared by the action of dicarbethoxycystinyl chloride on an alkaline solution of arginine. The esters of tyrosine and histidine were prepared by methods described in the literature.

Several different physiological tests (including the effect on blood sugar) of the above polypeptides have not yet been completed<sup>3</sup>.

---

<sup>3</sup>The compounds are being tested in the laboratories of Parke-Davis and Company.





# THE EFFECTS OF MOLYBDENUM AND CHROMIUM ON THE MALLEABILIZATION OF WHITE CAST IRON<sup>1</sup>

EVERETTE LEE HENDERSON

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

The present investigation was undertaken with the object of determining the quantitative effect of molybdenum and chromium, separately and in combination, upon the rates of graphitization of white cast iron.

## EXPERIMENTAL

### A. PREPARATION AND CHEMICAL COMPOSITION OF THE ALLOYS

Seventeen alloys were prepared from a commercial white cast iron base. Five of these alloys contained from one to five per cent molybdenum by weight. Nine of these alloys contained combinations of molybdenum and chromium from one to five per cent of each element. The remaining three alloys contained one, three and five per cent of chromium and no molybdenum.

Five thousand grams of the commercial cast iron were melted in a plumbago crucible by means of a 35 kv. induction furnace. The calculated quantities of ferro-molybdenum or ferro-chromium were added to the molten metal. Small amounts of powdered graphite and ferro-silicon were added to compensate for the burning out of these elements at the high temperature. The molten alloy was stirred with a pure iron rod just before pouring to assure a homogeneous composition. The alloys were cast into sand moulds at a pouring temperature of approximately 1350°C. and were allowed to cool to room temperature before breaking open the moulds.

The chemical composition of the white cast iron base and the molybdenum alloys is given in table 1.

TABLE 1

Melt No.	Weight percentage					
	Mo	C	Si	Mn	S	P
S-1-1	0.922	2.79	0.781	0.21	0.033	0.148
S-6-2	1.85	2.52	0.806	0.20	0.037	0.151
S-7-3	3.07	2.63	0.644	0.19	0.037	0.150
S-14-4	4.10	2.58	0.650	0.21	0.035	0.149
S-15-5	4.96	2.61	0.615	0.20	0.033	0.151
Com. Cast	0	2.61	0.781	0.20	0.033	0.154

No analysis of the chromium containing alloys was made because it was learned early in the investigation that graphitization was completely stopped in alloys containing as low as one per cent of chromium.

Samples of all compositions were found to be extremely hard as cast and some difficulty was experienced in preparing them for chemical analysis.

<sup>1</sup>Original Thesis submitted July, 1931.

## B. HEAT TREATMENT

As a preliminary operation, one inch samples of each composition were placed in cast iron pipe containers. The packing material was twenty mesh gas carbon. The samples and containers were placed in a Hump electric annealing furnace at 932°C. A container, with a sample of each composition, was removed at the following intervals:

½ hour, 10 hours, 48 hours, 100 hours, 74 hours, 257 hours

Upon removal at the stated intervals, the samples were ground down to a depth of about three-sixteenths inch. They were then polished and examined microscopically, both etched and unetched, to determine the progress of the decomposition of the massive cementite. A five per cent solution of concentrated nitric acid in ethyl alcohol was employed as the etching solution.

As soon as the approximate time for the primary stage of graphitization was determined, the above procedure was repeated until the exact time was found for each composition at the elevated temperature of 932°C. At least four samples of each composition was given the predetermined treatment.

A similar procedure was undertaken to determine the minimum time required for the secondary stage at 704°C. The samples, which had previously been given the required treatment at 932°C., were placed in the furnace at 932°C. and held at this temperature for one-half hour. The furnace was then cooled to 704°C. at a controlled rate of 18.5°C. per hour. This temperature was then maintained and samples were removed at twenty-four hour intervals and examined microscopically to determine the progress of the secondary graphitization stage. After the proper time had been found, all samples were returned to the furnace at proper intervals to insure that all samples of the same composition would have the same time at 704°C. When the proper time had elapsed, the furnace was cooled under control to 450°C. at a rate of 6.8°C. per hour. The samples were then removed from the furnace and air cooled in the carbon pack.

The samples were then ready for final microscopical examination and photographic study.

## PRESENTATION OF RESULTS

The following table shows the optimum time required for the completion of the primary and secondary stages of graphitization. Percentages of molybdenum are given in round numbers.

TABLE 2

Alloy no.	Weight percentage		Time for complete graphitization	
	Mo	Cr	At 932°C.	At 104°C.
S-1-1	1.00	0	75 to 80 hrs.	125 hrs.
S-6-2	2.00	0	90 to 100 hrs.	160 to 165 hrs.
S-7-3	3.00	0	150 to 155 hrs.	195 to 200 hrs.
S-13-4	4.00	0	200 to 205 hrs.	245 to 250 hrs.
S-14-5	5.00	0	Incomp. 255 hrs.	Incomp. 250 hrs.
X-9	1.00	1.00	No effect 406 hrs.	No effect 255 hrs.
X-13	0	1.00	No effect 406 hrs.	No effect 255 hrs.
Com. Cast	0	0	60 to 65 hrs.	100 to 105 hrs.

These results were obtained by examination of the samples under the microscope and by a study of the micro-photographs. Photographs were made of the samples at intermediate stages of graphitization and of the completely graphitized alloys. These show that the molybdenum serves to refine the grain structure. This partially explains the increased hardness and toughness of those alloys which contain molybdenum.

### CONCLUSIONS

Molybdenum, when present up to five per cent in white cast iron, exhibits an inhibiting effect upon the decomposition of the iron carbide, but does not completely stop the graphitization process. Both stages of graphitization show a decreasing rate with increasing quantity of molybdenum as indicated by a smooth curve which results by plotting the time required for completion of the graphitization against the percentage of molybdenum in the alloys.

Chromium has been found to completely stop the decomposition of the iron carbide under a heat treatment of 406 hours at 932°C. and 255 hours at 704°C.

The actual requirements as regards the necessary heat treatment for completion of graphitization are given in table 2.

No attempt has been made to correlate the physical properties with the chemical composition except the observations that were made during the breaking, grinding and polishing of the samples. Such observations have led to the belief that molybdenum imparts increased hardness, toughness and tensile strength to the malleablized iron. The alloys show a pronounced refinement of grain structure.

### SUMMARY

A study of the quantitative effects of molybdenum, chromium and a combination of these two elements up to five per cent, on the stability of cementite has been made.

2. Rates of the first and second stages of graphitization have been determined for alloys containing from one to five per cent of molybdenum.

3. It has been shown that the presence of chromium completely stops the graphitization process.





# FURFURAL AND SOME OF ITS DERIVATIVES<sup>1</sup>

AMIOT P. HEWLETT

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

## INTRODUCTION

Furfural may be secured by the acid hydrolysis of practically all vegetable matter. Farm waste products, therefore, are the chief sources of this substance. At the present time, the furfural of commerce is largely prepared from oat hulls. The annual possible production of furfural in this country, from farm waste products, is practically unlimited. The total available quantity from oat hulls and corncobs alone is about 1,104,200,000 pounds per year, while the total production for the year 1929 was only 1,500,000 pounds. Therefore, the production of furfural depends only on the demand for that substance. At the present time, furfural is being used as a substitute for the more expensive formaldehyde in the preparation of resins of the bakelite type, as insecticides and fungicides and as an embalming fluid.

This is a part of the work being done at Iowa State College on utilization of agricultural waste products. In this work the problem of utilization of furfural is being attacked from the point of view of chemistry. This consists of the preparation of furfural derivatives, which are to be used in the synthesis of more valuable and more useful compounds and also the synthesis of antiseptics and dyes.

## EXPERIMENTAL

*5-Bromofurylacetenylmagnesium Bromide.* This compound was prepared by the addition of 5-bromofurylacetylene<sup>2</sup> to a solution of ethylmagnesium bromide in ether. Treatment of the resulting solution with carbon dioxide gave the corresponding 5-bromofurylpropionic acid in yields of 21.8 per cent, while treatment with  $\alpha$ -naphthyl isocyanate gave the corresponding  $\alpha$ -naphthalide, melting at 150°, in yields of 35 per cent.

The 5-bromofurylacetylene was prepared by bromination of furylacryloyl chloride<sup>3</sup> to produce 5-bromofurylbromoacryloyl chloride in yields of 96 per cent (crude) boiling at 90°-95°/24 mm. and melting at 69°-70°. The pure compound boils at 182°-183°/21 mm. and melts at 72°. The 5-bromofurylbromoacryloyl chloride was hydrolyzed with dilute sodium hydroxide to yield 69.5 per cent (crude) of 5-bromofurylpropionic acid melting at 127°. The pure acid melts at 143°. The crude 5-bromofurylpropionic acid was subjected to steam distillation to produce 29.4 per cent of 5-bromofurylacetylene boiling at 63°-64°/24 mm. This compound was con-

<sup>1</sup>Original Thesis submitted December, 1931.

<sup>2</sup>Gibson and Kahnweiler, *Am. Chem. J.*, **12**, 314 (1890).

verted to the diacetylene, melting at  $126^{\circ}$ , which agrees with the value reported in the literature<sup>2</sup>.

*Furyl-Alkyl Halides.* 1-Furyl-3-chloropropane and 1-tetrahydrofuryl-3-chloropropane were prepared by the action of thionyl chloride on the corresponding alcohols. The 1-furyl-3-chloropropane was prepared in yield of 20 per cent from furylpropyl alcohol<sup>4</sup> by the method used by Kirner<sup>5</sup> in the preparation of  $\alpha$ -furfuryl chloride. This compound boils at  $60^{\circ}/5$  mm. and is a colorless liquid,  $d_{25}^{25}$  1.0813;  $n_D^{25}$  1.4730.

*Analysis.* Calculated for  $C_7H_9OCl$ : Cl, 24.57. Found: Cl, 24.49 and 24.73.

The 1-tetrahydrofuryl-3-chloropropane is best prepared by addition of one mole of thionyl chloride to a boiling solution of one mole of the tetrahydrofurylpropyl alcohol<sup>4</sup> in benzene. The yield of the chloride boiling at  $75^{\circ}/4$  mm. was 82.5 per cent. This compound is a colorless liquid,  $d_{25}^{25}$  1.0425;  $n_D^{25}$  1.4540.

*Analysis.* Calculated for  $C_7H_{13}OCl$ : Cl, 23.90. Found: Cl, 23.74 and 23.76.

The 1-furyl-3-chloropropane did not react with magnesium in some preliminary tests, while the 1-tetrahydrofuryl-3-chloropropane reacted vigorously to form the corresponding Grignard reagent in yields of 91 per cent, by acid titration. This Grignard reagent was treated with carbon dioxide to yield 47.5 per cent of tetrahydrofuryl-*n*-butyric acid boiling at  $145^{\circ}/5$  mm. This acid is a colorless liquid with an unpleasant odor,  $d_{25}^{25}$  1.2286;  $n_D^{25}$  1.4572; neutralization equivalent 165, the calculated value being 158.

*Analysis.* Calculated for  $C_8H_{14}O_3$ : C, 60.76; H, 8.86. Found: C, 60.89 and 60.72; H, 8.66 and 8.69.

The 1-tetrahydrofuryl-3-chloropropane was converted quantitatively into tetrahydrofurylpropyl thiocyanate by heating a mixture of one mole of the chloride in 500 cc. of alcohol and 1.5 moles of potassium thiocyanate in a sealed tube at  $120^{\circ}$  for 12 hours. Tetrahydrofurylpropyl thiocyanate is a colorless liquid boiling at  $138^{\circ}/8$  mm.,  $d_4^{30}$  1.0660;  $n_D^{30}$  1.4890.

*Analysis.* Calculated for  $C_8H_{13}ONS$ : S, 18.71. Found: S, 18.93.

The 1-tetrahydrofuryl-3-chloropropane was converted into tetrahydrofurylpropyl mercaptan in yields of 34 per cent by heating a mixture of equimolecular proportions of the chloride and thiourea and treating the resulting product with ammonium hydroxide. The mercaptan is a liquid with an extremely offensive odor, boiling at  $85^{\circ}/10$  mm.,  $d_4^{25}$  1.0006;  $n_D^{34}$  1.4807.

*Analysis.* Calculated for  $C_7H_{14}OS$ : S, 21.91. Found: S, 21.60.

*5-Chlorofuroic Acid.* This compound was formerly prepared by Hill and Jackson<sup>6</sup>. 5-Chlorofuroyl chloride was prepared by treatment of furoyl chloride with chlorine at a temperature of  $100^{\circ}$ . This compound was secured in yields of 66 per cent, boiling at  $92^{\circ}$ - $95^{\circ}/10$  mm. and was converted to the corresponding amide which melted at  $154^{\circ}$ , which is in agreement with the value reported elsewhere<sup>6</sup>. The chloro-amide was hydrolyzed to yield 54 per cent of 5-chlorofuroic acid melting at  $177^{\circ}$ .

<sup>2</sup>Gilman and Hewlett, *Iowa State College Jour. Science*, **4**, 31 (1930).

<sup>4</sup>Adams and Bray, *J. Am. Chem. Soc.*, **59**, 2101 (1927).

<sup>5</sup>Kirner, *J. Am. Chem. Soc.*, **50**, 1955 (1928).

<sup>6</sup>Hill and Jackson, *Am. Chem. J.*, **12**, 22 (1890).

*Lachrymators of the Furan Series.* The lachrymatory power of furoyl chloride, 5-chlorofuroyl chloride, tetrahydrofuryl iodoacetate and tetrahydrofuryl chloroacetate has been determined. While these substances are lachrymators, they are less effective than chloroacetophenone. The tetrahydrofuryl chloroacetate was prepared in yields of 99 per cent, boiling at  $110^{\circ}/5$  mm. by addition of one mole of chloroacetyl chloride to a boiling benzene solution of one mole of tetrahydrofurfuryl alcohol. This compound was previously prepared by Gilman and Dickey<sup>7</sup>. The iodoacetate was prepared by heating a solution of the chloroacetate in alcohol with sodium iodide. The yield of iodoacetate boiling at  $130^{\circ}/5$  mm. was 65 per cent.

*Analysis.* Calculated for  $C_7H_{11}O_3I$ : I, 47.21. Found: I, 47.14.

*Antiseptics.* The phenolic esters of furoic acid were prepared by the Schotten-Baumann reaction, using furoyl chloride and an alkaline solution of the phenol, with the exception of catechol and hydroquinone furoates, which were prepared by heating a mixture of the acid chloride and the phenolic compound. The esters of furylacrylic acid were prepared by refluxing a benzene solution of the acid chloride and the phenolic compound. The full ester was always secured in the case of the furoates, while the half esters were secured in the case of the furylacrylates, with the exception of resorcinol furylacrylate. In the case of this compound, either the half or full ester was secured, depending upon the quantity of furylacryloyl chloride used. Tetrahydrofurfuryl oxalate was prepared by heating anhydrous oxalic acid with a 200 per cent excess of the alcohol. Tetrahydrofurylpropyl oxalate was prepared by treating the sodium salt of the alcohol in benzene with equimolecular proportions of oxalyl chloride. Tetrahydrofurfuryl salicylate was prepared by refluxing a solution of salicyloyl chloride<sup>8</sup> and tetrahydrofurfuryl alcohol in benzene. The esters were all hydrolyzed and the acids identified by mixed melting point. In cases where the composition of the ester was in doubt, verification was effected by determination of saponification equivalent and the melting points of mixtures of the ester with the acid and also with the phenol.

A study of the antiseptic property of these compounds is now being made. The results will be reported at a later date.

*Insecticides of the Furan Series.* The insecticide property of a number of furan compounds has been studied. They were all found to be relatively inactive, with the exception of tetrahydrofurylpropyl thiocyanate, benzoylfuran<sup>3</sup> and the *p*-cresol and phenyl esters.

*Furfural Diacetate.* Following the method of Gilman and Wright<sup>9</sup> it was found possible to secure furfural diacetate in yields of 60-70 per cent by the use of one gram of stannous chloride per mole of furfural and allowing the reaction mixture to stand in an ice box for 48 hours. Also, yields of 50 per cent were secured by addition of one mole of acetic anhydride to a solution of two grams of stannous chloride in one mole of furfural at a temperature of  $20^{\circ}$ - $30^{\circ}$ . The mixture was then stirred for two hours, filtered, crystallized from carbon disulphide and distilled.

<sup>7</sup>Gilman and Dickey, Unpublished results.

<sup>8</sup>Adams and Ulich, *J. Am. Chem. Soc.*, **42**, 604 (1920).

<sup>9</sup>Gilman and Wright, *Iowa State College Jour. Sci.*, **4**, 35 (1929).

TABLE 1. *Data on esters*

No.		M. p. °C.	B. p. °C.	P. mm.	Yield Pctg.
1	Phenyl furoate	42	145	44	94
2	Phenyl furylacrylate	.....	185	4	84
3	Guaiacol furoate	76	175	5	82
4	Guaiacol furylacrylate	105	210	6	70
5	<i>p</i> -Cresol furoate	55	152	5	64
6	<i>p</i> -Cresol furylacrylate	75	195	6	71
7	<i>m</i> -Cresol furoate	40	155	5	90
8	<i>m</i> -Cresol furylacrylate	.....	185	5	61
9	Resorcinol furoate	130	.....	....	37
10	Catechol furoate	116	.....	....	34
11	Hydroquinone furoate	200	.....	....	27
12	Resorcinol furylacrylate	128	.....	....	35
13	Hydroquinone furylacrylate	173	.....	....	30
14	Catechol furylacrylate	132	.....	....	35
15	Resorcinol di-furylacrylate	112	.....	....	95
16	Tetrahydrofurfuryl oxalate	.....	203	4	58
17	Tetrahydrofurylpropyl oxalate	.....	210	3	51
18	Tetrahydrofurfuryl salicylate	.....	166	5	78

## Analyses

No.	Carbon, Pctg.		Hydrogen, Pctg.		Saponifi- cation equivalent
	Calcd.	Found	Calcd.	Found	
1	70.21	70.16	4.25	4.42	225
2	72.88	72.78	4.67	4.87	
3	66.05	65.92	4.69	4.74	
4	68.85	69.23	4.92	5.12	
5	71.28	71.66	4.95	4.82	
6	73.68	73.56	5.26	5.31	
7	71.28	70.95	4.95	4.90	147
8	73.68	73.96	5.26	5.39	
9	64.43	64.10	3.35	3.19	
10	64.43	64.73	3.35	3.61	
11	64.43	64.59	3.35	3.81	
12	67.83	67.95	4.35	4.50	
13	67.83	67.50	4.35	4.34	208
14	67.83	67.76	4.35	4.72	208
15	68.57	69.02	4.00	4.23	165
16	55.81	55.79	6.98	7.21	127
17	61.15	61.00	8.25	8.14	157
18	63.26	63.25	5.77	5.92	



## PREPARATION OF SECONDARY AND TERTIARY FURYL CARBINOLS

Following the work of Hale, McNally and Pater<sup>10</sup> and of Peters and Fischer<sup>11</sup>, respectively, diphenyl furyl and phenyl furyl carbinols were prepared. These compounds are unstable, but seem to be more stable than was formerly indicated. Di-*tert.* butyl furyl carbinol was prepared by the action of ethyl furoate and also furoyl chloride on *tert.* butylmagnesium chloride. The di-*tert.* butyl furyl carbinol was not secured in the pure condition, analyses indicating that the substance was a mixture. This material, however, is quite stable. 5-Chlorofuryl and 5-bromofuryl carbinols were prepared by the action of the corresponding aldehyde on phenylmagnesium bromide. These compounds undergo decomposition on standing, with elimination of halogen acid and with the formation of a small amount of a stable, halogen free, compound melting at 86°. The same substance is secured from both of the above mentioned compounds. The 5-bromofurfural was prepared by the method of Gilman and Wright<sup>12</sup>. The 5-chlorofurfural was prepared by the action of sulfuryl chloride on a solution of furfural diacetate in carbon disulphide according to the method of Gilman and Wright<sup>13</sup>.

Di-*tert.* butyl furyl carbinol is a yellow liquid boiling at 80°85°/1 mm.,  $d_4^{24}$  0.9486;  $n_D^{24}$  1.4749. The yield was 25 per cent.

*Analysis.* Calculated for  $C_{13}H_{22}O_2$ : C, 74.29; H, 10.48. Found: C, 73.56 and 73.45; H, 9.87 and 9.73.

5-Chlorofuryl phenyl carbinol is a white solid melting at 113°, yield 24 per cent.

*Analysis.* Calculated for  $C_{11}H_9O_2Cl$ : Cl, 17.07. Found: Cl, 17.28 and 17.19.

The 5-bromofuryl phenyl carbinol is a white solid melting at 128°, yield 23.6 per cent.

*Analysis.* Calculated for  $C_{11}H_9O_2Br$ : Br, 24.69. Found, Br, 24.38 and 24.11.

*Dyes of the Furan Series.* *p*-Dimethylaminophenylmagnesium bromide was prepared in yields of 40-45 per cent, based on acid titration, by the action of *p*-bromodimethylaniline on magnesium in the presence of one equivalent of "magnesium iodide." This Grignard reagent was treated with ethyl furoate to prepare the furyl analog of malachite green, formerly described by Renshaw and Naylor<sup>14</sup>. The yields were very unsatisfactory and no stable derivatives of the carbinol base were secured. The furyl analog was also prepared by the method of Renshaw and Naylor<sup>14</sup> and the only comparison made between the compounds from the two sources was that of the color produced on fabric and the analyses of the platinum salts.

<sup>10</sup>Hale, McNally and Pater, *Am. Chem. J.*, **35**, 68 (1906).

<sup>11</sup>Peters and Fischer, *J. Am. Chem. Soc.*, **52**, 2079 (1930).

<sup>12</sup>Gilman and Wright, *J. Am. Chem. Soc.*, **52**, 1170 (1930).

<sup>13</sup>Unpublished results.

<sup>14</sup>Renshaw and Naylor, *J. Am. Chem. Soc.*, **44**, 862 (1922).

The 5-bromofuryl, 5-chlorofuryl and nitrofuryl analogs were prepared by the condensation of the corresponding aldehyde with dimethylaniline in the presence of zinc chloride. In the case of the nitrofuryl analog, no condensation agent was necessary, the mixture merely being allowed to stand for one week. The nitrofurfural was prepared by the method of Gilman and Wright<sup>15</sup>. The leuco bases were oxidized according to the usual method for malachite green<sup>14</sup> and the zinc chloride double salts were prepared and found to be stable dyes, producing blue colors on silk, artificial silk, wool and mordanted cotton. The platinum salts of the carbinol bases were prepared and analyses for platinum found to agree with the theoretic values. The furyl analog, leuco base, was secured in yields of 6.9 per cent melting at 87°; the 5-bromofuryl analog in yields of 12.9 per cent melting at 132°; the 5-chlorofuryl analog in yields of 14.1 per cent melting at 117°; and the nitrofuryl analog in yields of 23.5 per cent melting at 135°.

*Analyses.* Calculated for  $C_{21}H_{23}BrN_2O$  (bromofuryl analog): Br, 20.05. Found: Br, 19.88. Calculated for  $C_{21}H_{23}ClN_2O$  (chlorofuryl analog): Cl, 10.01. Found: Cl, 10.11 and 10.09. Calculated for  $C_{21}H_{23}N_3O_3$  (nitrofuryl analog): C, 69.04; H, 6.30. Found: C, 68.66 and 68.93; H, 6.27 and 6.42.

5-Bromofurfural, 5-chlorofurfural and nitrofurfural were treated with a mixture of aniline and aniline hydrobromide in an attempt at preparation of the analogous hydroxyglutaconaldehydedianilides and 3-hydroxy-1-phenylpyridinium salts of Stenhouse and others<sup>16</sup>. It was found impossible to secure the nitro analog, complete decomposition resulting. However, the same identical substance was secured from both 5-bromofurfural and 5-chlorofurfural when treated with a mixture of aniline and aniline hydrobromide. Analyses show that the halogen substituent of the ring is lost during the condensation. This compound melts at 145° and was secured from both sources in yields of 63 per cent. This compound is yellow in color, slightly soluble in water and dyes silk, artificial silk, wool and cotton in a golden yellow color which is very fast. This compound is not converted into the pyridinium salt by the methods used for the conversion of the compound of Stenhouse and others<sup>16</sup>. For this reason, the structure suggested for this substance is  $PhN=C=CH-CH=C(OH)-CH=NPh \cdot HBr \cdot H_2O$ .

*Analysis.* Calculated for  $C_{17}H_{15}BrN_2O \cdot H_2O$ : Br, 22.16. Found: Br, 22.16 and 21.94.

<sup>15</sup>Gilman and Wright, *J. Am. Chem. Soc.*, **52**, 2550 (1930).

<sup>16</sup>Stenhouse, *Ann.*, **155**, 199 (1870); Zireke and Mulhausen, *Ber.*, **33**, 3824 (1905); König, *J. prakt. Chem.*, **63**, 105 (1904); *ibid.*, **72**, 555 (1905); Dieckmann and Beck, *Ber.*, **32**, 4122 (1905).

## SUMMARY

5-Bromofurylacetenylmagnesium bromide has been prepared and characterized by conversion to the corresponding acid and  $\alpha$ -naphthalide.

1-Furyl-3-chloropropane and 1-tetrahydrofuryl-3-chloropropane have been prepared and described. The latter reacts with magnesium to form the Grignard reagent, and was also converted to the corresponding thiocyanate and mercaptan.

5-Chlorofuroyl chloride has been prepared and described. This compound was converted to the corresponding acid and amide.

The lachrymatory properties of a group of furan compounds have been studied.

The furyl and furylacrylic esters of phenol, guaiacol, *p*-cresol, *m*-cresol, resorcinol, catechol and hydroquinone and the tetrahydrofurfuryl and tetrahydrofurylpropyl esters of oxalic acid and the tetrahydrofurfuryl esters of salicylic acid have been prepared and described.

Di-*tert.* butyl furyl, 5-bromofuryl phenyl and 5-chlorofuryl phenyl carbinols have been prepared and described.

The 5-bromofuryl, 5-chlorofuryl and nitrofuryl analogs of malachite green have been prepared and described. A condensation product of 5-chloro and 5-bromofurfural with aniline and aniline hydrobromide has been prepared and described.





# A STUDY OF SOME OF THE LACTOBACILLI<sup>1</sup>

LINCOLN SPENCER HYDE

*From the Department of Dairy Industry, Iowa State College*

Accepted for publication June 15, 1932

Certain members of the genus *Lactobacillus* (Beijerinck) are of particular importance to the dairy industry; namely, *L. acidophilus* (Moro), *L. bulgaricus* (Grigoroff), and *L. casei* (v. Freudenreich). The first two have in recent years occasioned increasing interest because of their suggested therapeutic value in the treatment of intestinal disorders and because they are so alike culturally that some doubt still exists as to definite differentiating characters. The third, although recognized several years prior to the others, has received comparatively little attention except in its relationship to the ripening of certain types of cheese.

This investigation of various organisms usually considered as belonging to these three species was made with a view to correlating some of the more outstanding characters, which were considered valuable in proper identification and classification.

## RESULTS OBTAINED

The 86 cultures studied were secured from various sources: 33 were from research and commercial laboratories and included 9 cultures of *L. acidophilus*, 7 *L. bulgaricus*, and 17 *L. casei*; 53 were isolated, 16 from the fecal matter of humans and animals were considered as *L. acidophilus*, while 37 from raw milk and cheddar cheese were considered as *L. casei*.

Since fewer failures resulted, the Heymann acetic acid bouillon method of isolating *L. acidophilus* strains from fecal material proved more certain than a dilution method or direct plating, which were apparently successful only under particularly favorable conditions.

Milk proved very satisfactory as an enrichment medium when isolating lactobacilli from milk and cheese. The frequency with which *L. casei* cultures were secured from milk and cheese showed their prevalence in these products; numerous investigators have reported similar results.

For convenience in discussion the organisms were classified into two groups according to the size of the cells; one designated as large, the other as small.

The ability of the cultures to grow at various temperatures seemed to show some correlation between this character and the size of the organisms. For the most part the cultures classed as large among the *L. bulgaricus* and *L. acidophilus* groups were able to grow at 45°C., but were unable to grow or grew poorly at room temperature and at 15-20°C. Conversely, the small cultures in these groups were unable to grow or grew poorly at 45°C., but were for the most part able to grow at the lower temperatures. Twelve large *L. casei* cultures failed to grow at any of these temperatures, while at 37°C. these cultures grew very slowly. Eleven *L. casei* cultures, all except one classed as small, grew at 45°C., but rather poorly, while prac-

---

<sup>1</sup>Original Thesis submitted June, 1927.

tically all except the twelve noted grew well at room temperature and at 15-20°C. It appears from these results that in general the large organisms grew better than the small at 45°C., while the small grew better than the large at the lower temperatures. Since *L. bulgaricus* has long been recognized as having a high optimum growth temperature these relationships seemed to be significant. Studies of other characters emphasized this relationship.

Thirteen trials were made using several different media and depressants to determine the effect of lowered surface tension of the medium on the growth of the organisms. Sodium ricinoleate was found to be superior as a depressant to sodium taurocholate, sodium glycocholate or sodium oleate so in most of the trials sodium ricinoleate was used. When the surface tension of the medium was depressed much under 40 dynes the first cultures failing to grow were apparently in the group classed as large. In medium X (Albus and Holm) all of the 60 cultures tested grew at 40 dynes; at 37.4 dynes 23 of the 82 cultures failed to grow—16 of these were large and 7 small. In whey peptone broth at 39 dynes, 9 of 87 cultures failed to grow and all were classed as large; at 37.3 dynes, 58 of 87 failed to grow and it was noticeable that the small *L. acidophilus* and *L. bulgaricus* strains were among the few which grew. In beef infusion bouillon 17 of 60 cultures failed to grow and 16 of these were classed as large. Among 72 cultures tried in medium M (medium X with maltose substituted for lactose), 4 large strains of *L. bulgaricus* and *L. acidophilus* failed to grow without depressant; at 39 dynes, 9 failed to grow and all were classed as large. From these results it is evident that a close relationship exists between the large *L. acidophilus* and *L. bulgaricus* organisms in respect to their ability to grow in media with reduced surface tensions. The results also indicate that 40 dynes is a critical surface tension for the *L. bulgaricus* types, and that the small *L. acidophilus* and *L. casei* types are able to grow at nearly 37 dynes. The suitability of the medium without depressant as well as the depressant used undoubtedly influence the ability of the organisms to grow at reduced surface tensions.

A study of the type of lactic acid produced in milk by 12 cultures considered as *L. acidophilus* showed variations from pure active acid of the dextro modification to practically pure inactive acid. Four of six large cultures produced largely or entirely inactive acid. In other characters, such as growth at 45°C., but not at 15-20°C., failure to grow in medium X at 37.4 dynes, and the failure of one of them to ferment lactose, these cultures closely resembled the *L. bulgaricus* cultures classed as large. These four were among five from other laboratories studied for the type of lactic acid produced.

Determinations of the total and volatile acids produced in milk by 66 cultures did not show any particularly striking relationships. Five of six cultures, two *L. acidophilus* from laboratory sources and three *L. bulgaricus*, producing over 2.0 per cent acid were classed as large, grew at 45°C., but not at 15-20°C., and failed to grow at the lower surface tensions. The volatile acidities varied widely, the values ranging from 4.7 to 41.7. The *L. acidophilus* cultures seemed to produce slightly higher volatile acidities since the values obtained with several were over 30, while none over 30 were obtained with the *L. bulgaricus* cultures and with only an occasional *L. casei* culture.

Sixteen cultures were studied as to their proteolytic activity in milk both with and without  $\text{CaCO}_3$  during an incubation of 30 days. All of the cultures caused some increase in soluble nitrogen; the values varied from 1.0 to 6.7 per cent in the milk without  $\text{CaCO}_3$ , and from 1.3 to 19.2 per cent in the milk with  $\text{CaCO}_3$ . In nearly all instances a culture caused higher increases in milk with than without the carbonate. When a culture caused the highest increase with  $\text{CaCO}_3$  it also caused the highest without  $\text{CaCO}_3$ . No particularly significant relationships seemed to exist between the amount of proteolysis and other characters.

These studies of the various characters of the organisms seemed to bring out correlations which would justify certain conclusions as to their inter-relationships. Most of the large cultures in the *L. acidophilus* groups exhibited characteristics which indicated close relationship to the large *L. bulgaricus* cultures. On the other hand, the small *L. acidophilus* types showed close relationship to the *L. casei* cultures since their reactions to the conditions mentioned have been pointed out as being opposite to those shown by the large types. It seems that many of the large types previously considered as *L. acidophilus* should have been considered as *L. bulgaricus* and the small types as *L. casei* or else that two types of *L. acidophilus* exist, one of which is closely related to *L. bulgaricus* and the other to *L. casei*. If the latter view is to be accepted, *L. casei* seems to bear closer relationships to both *L. acidophilus* and *L. bulgaricus* than these two species do to one another. If any one species is to be considered a central type, it appears from this study that *L. casei* rather than *L. acidophilus* should be accepted as the central type. In any case it must be recognized that certain forms are found which apparently are borderline strains lying between two species so that no absolutely definite line of demarcation can be drawn between them. The close relationship between the *L. casei* and *L. acidophilus* types suggested the probability that *L. casei* could be implanted in the intestinal tract since indications are that *L. acidophilus* can be.

Feeding experiments were conducted with four young men and with rats, the effects being determined by bacteriological examination of the feces.

The men consumed daily, with the regular diet, one quart of milk fermented with several cultures of *L. casei* isolated from milk and cheese. The rats were fed suspensions of pure cultures of single strains of *L. casei* or *L. acidophilus* with a basal diet of fresh white bread and fresh beef, with lactose added except in the case of the control rats during the latter part of the trials. Results obtained seemed to justify the conclusion that transformation of the intestinal flora took place with the men. The change in flora with the rats was less definite although sufficient for an unbiased observer to detect microscopically which fecal smears were from rats receiving cultures and which were from the controls. No such proportions of Gram-positive organisms were found in any fecal smears as have been reported by several investigators. The results were more in agreement with those reported by Kopeloff (1926).

The ability to become implanted in the intestinal tract further emphasized the close relationship between *L. casei* and *L. acidophilus*, particularly between the small strains. It also indicated the probability that many reports of favorable changes in the intestinal flora from milk feeding have been due in part at least to the presence of *L. casei* types

in the milk. The importance to the dairy industry of the prevalence of these types in milk and cheese is further brought out by the results of these trials.

A satisfactory white acidophilus milk was produced by two exposures of a good quality milk at 195-200°F. for one hour each, with an incubation between of three hours at 100°F., although large Gram-positive rods very evidently not *L. acidophilus* were present in every instance. This method was not as consistently successful as when the milk was sterilized.

Storage of acidophilus milk at refrigerator temperature was found to be more satisfactory than at room temperature both from the standpoint of palatability and the number of organisms remaining viable after seven days. The organisms were also found to be able to survive freezing in the form of lacto; the average of six trials showed that 56.7 per cent of the original number were viable after seven days. This was considered a new and satisfactory medium for supplying viable *L. acidophilus* to persons who would not take acidophilus milk in the usual form.

#### BIBLIOGRAPHY

ALBUS, W. R., AND G. E. HOLM

1925. The effect of surface tension on the growth of *L. acidophilus* and *L. bulgaricus*. *Proc. Soc. Exp. Biol. Med.*, **22**:337-338. Also

1926. *Jour. Bact.*, **12**:13-18.

FREUDENREICH, E. VON

1891. Bakteriologische Untersuchungen ueber den Reifungs prozess des Emmen-thalerkäse. *Landw. Jahrb. Schweiz.*, **5**:16-29.

---

1899. Ueber die Beteiligung der Milchsäurebakterien an der Käsereifung. *Centbl. Bakt.*, 2 abt. **5**:241-249.

---

UND J. THÖNI

1904. Ueber die Wirkung verschiedener Milchsäurefermente auf die Käsereifung. *Landw. Jahrb. Schweiz.*, **18**:531-558.

GRIGOROFF, S.

1905. Étude sur un lait fermenté comestible. Le "kisselo-mléko" de Bulgarie. *Rev. Méd. d. l. Suisse romande.*, **25**:714-721.

KOPELOFF, N.

1926. *Lactobacillus acidophilus*. The Williams and Wilkins Co., Baltimore.

MORO, E.

1900. Ueber den *Bacillus acidophilus*. n. sp. *Jahrb. f. Kinderh.*, **52**:38-55.

MORTENSEN, M., AND J. GORDON

1911. Lacto: a new and healthful frozen dairy product. *Ia. Agr. Exp. Sta. Bul.* **118**.

---

AND HAMMER, B. W.

1913. Lacto: a frozen dairy product. *Ia. Agr. Exp. Sta. Bul.* **140**.



# STUDIES IN VITAMIN A TECHNIC<sup>1</sup>

MARGARET HOUSE IRWIN

*From the Department of Foods and Nutrition, Iowa State College*

Accepted for publication June 15, 1932

An application of statistical method to the data of 469 vitamin A feeding tests was made with a view to determining the factors influencing the weight-gains of the test-animals. The data of this study indicated a possible difference in the reactions of rats fed vitamin A-free diets containing different amounts of fats. These data showed also that the quantity of basal diet ingested was the measured variable having the greatest percentage effect upon the weight-gains of the animals.

An experiment was conducted to test the difference in the reactions during the depletion period of vitamin A test animals fed diets containing and not containing fat. One hundred and twenty-three animals were fed the basal diet containing fat and 60 animals the fat free basal diet. The mean initial weight, mean gain, mean days to depletion, and mean basal diet eaten daily for each of these two groups of animals are recorded in table 1.

The difference in the mean gains in weight was not found to be significant, but the difference in the number of days to depletion was significant. These data show that a vitamin A-free basal diet containing fat is preferable to a fat-free basal diet as it shortens the depletion period.

An analysis of the reactions of vitamin A test animals from three different stock colonies was made. One of these colonies came from the Chemistry Department of Iowa State College and was maintained in this laboratory without an organized scheme of mating. The second colony was obtained from the Wistar Institute. These animals were from a strain that had been inbred for fifty generations and were maintained in this laboratory by strictly brother and sister matings. The data for the third colony were very kindly furnished by Dr. Hazel E. Munsell of the Bureau of Home Economics, U. S. D. A., Washington, D. C. The difference in the mean in-

TABLE 1

	Osborne & Mendel vitamin A-free diet containing fat	Sherman & Munsell vitamin A-free diet containing no fat
Mean initial weight, gms.	51.9 $\pm$ 0.29	55.3 $\pm$ 0.82
Mean gain during depletion, gms.	70.2 $\pm$ 0.87	74.6 $\pm$ 2.00
Mean days to depletion, days	36.0 $\pm$ 0.33	39.0 $\pm$ 0.46
Mean basal diet eaten daily, gms.	8.66 $\pm$ 0.06	10.67 $\pm$ 0.12
Mean calories eaten daily, calories	40.7	38.4

<sup>1</sup>Original Thesis submitted July, 1931.

itial weights, mean gains in weight, mean days to depletion and mean daily food intake during the depletion period of vitamin A test animals from these three colonies were found to be significant. These data show that the results of a study using animals of one colony could not be applied directly to the data of another colony without first testing the homogeneity of the two groups of animals.

A statistical treatment of the data of the vitamin A depletion period of 123 standard test animals from the Wistar colony was made and upon the basis of the knowledge thus gained an attempt was made to control the weight-gains of the animals by controlling the amount of basal diet they consumed. The method used was designed to produce animals whose weights at the end of the depletion period approach more closely the mean than the weights of animals fed the basal diet ad libitum.

The means and probable errors for the depletion period of these 123 animals used for vitamin A tests in this laboratory are:

Mean initial weight .....	51.9 ± 0.29
Mean gain .....	70.2 ± 0.87
Mean days to depletion .....	35.9 ± 0.33
Mean basal diet eaten daily .....	8.66 ± 0.06

The correlations between the variables are:

Initial weight and total gain .....	0.24 ± 0.06
Initial weight and days to depletion .....	0.53 ± 0.04
Initial weight and mean basal diet eaten daily .....	0.27 ± 0.06
Total gain and days to depletion .....	0.31 ± 0.05
Total gain and mean basal diet eaten daily .....	0.64 ± 0.04
Days to depletion and mean basal diet eaten daily .....	0.09 ± 0.06

The largest and most significant of these correlations is that between the total gain and the mean basal diet eaten daily,  $0.64 \pm 0.04$ . A multiple correlation coefficient (R), and a regression equation were calculated. The initial weight (A), total gain (B), and days to depletion (C), were used as the independent variables and the mean daily food intake ( $\bar{X}$ ) as the dependent variable. The multiple correlation coefficient (R) and the regression equation for this group of 123 animals are:

$$R = 0.77 \pm 0.04$$

$$\bar{X} = 0.07A + 0.05B - 0.09C + 4.56$$

By substituting in the regression equation an estimate of the amount of food to be fed to each animal was made in advance and the animal fed according to a food intake curve based upon the food consumption records of the original 123 animals.

Five groups of twenty rats each were fed. The first group was fed the basal vitamin A free diet ad libitum to serve as a control group. The

# THE DEVELOPMENT OF SYNTHETIC LUMBER FROM CORNSTALKS

ROGER W. RICHARDSON

*From the Department of Chemical Engineering, Iowa State College*

Accepted for publication June 15, 1932

## SMALL SCALE EXPERIMENTS

The Method: (1) Subjecting the shredded stalks to a water or chemical digestion or to a mechanical defibering treatment or to both; (2) forming a mat of pulp from the water suspension by removing the water in a suction mold; (3) applying pressure to the wet mat, and (4) drying in a shelf dryer. After a proper seasoning period, the boards were tested.

*Sizing.* Three per cent was found to be the optimum pulp consistency, but in commercial practice this factor would depend somewhat on the board-forming machine used. From 40 to 60°C. was found to be the best temperature range for sizing and for the formation of good board. Different kinds of water had little effect upon sizing.

*Effect of vacuum.* The vacuum for best board formation was found to be from 12 to 18 inches of mercury.

*Relation of various properties.* The strength of the boards was almost directly proportional to a decrease in freeness of the pulp, and to an increase in the density. The water absorption did not vary with the freeness. It would be expected that the modulus of rupture increased with the increase in pressure applied in forming the board, but this relationship is not sharply defined. The stronger and denser boards were slightly more resistant to water penetration.

*Drying experiments.* The range of best drying temperature was found to be 340-360°F.

*Miscellaneous experiments.* Thermal conductivity of both mechanical and cooked pulp insulating board was found to vary with density; an almost straight line relationship existing. Boards made of several types of raw material showed widely different coefficients of thermal conductivity.

Old stalks gave yields of pulp from four to six per cent higher than freshly harvested stalks, the stored stalks having lost soluble materials during storage. The loss of cooking for three hours in water at 45 pounds pressure was found to be about 20 per cent.

## SEMI-COMMERCIAL SCALE WORK

Process Developed: Baled stalks were broken open and dry-shredded with an ensilage cutter. The shredded material was cooked in water (rotary digester) for two hours at 90 pounds steam pressure, refined in a rod-mill, followed by washing in a trommel washer, and further refined in a Claflin refiner. Rosin size was added before the Claflin, and alum immediately after. The mat was formed on a forming machine and pressed between three sets of press rolls. The board was dried in a Coe roller dryer.

---

\*Original Thesis submitted June, 1930.

A yield of 55 per cent based on bone dry weight of stalks may be expected by the above process. A yield of as high as 77 per cent was obtained by a strictly mechanical process.

Pulp cooked with chemicals was found to be less desirable than water cooked pulp. The rod-mill-Claffin combination was the best means of pulp refining investigated.

Board made with a mixture of mechanical and cooked pulp resembled mechanical rather than cooked pulp board, although as much as six per cent of scrap and trim may be added to the pulp without causing a noticeable decrease in quality. Up to ten per cent of newsprint mixed with mechanical pulp improved the board flexibility. Added to cooked pulp, it resulted in a mixture difficult to handle on a forming machine. Up to 50 per cent of straw pulp can be satisfactorily used with cooked cornstalk pulp. Broom corn may also be used, but requires more strenuous cooking. Fair types of board can be made from artichoke plants, milk weeds and flax straw.

Overcooking of stalks results in a lowering of the quality of product; undercooking in a product resembling mechanical pulp. Allowing cooked pulp to age up to a period of three days improves the quality very slightly. There would be some advantage in forming boards at a stock temperature of from 40 to 60°C.

It is possible to use up to one per cent of sulphuric acid in sizing to reduce alum costs. It is best to add the acid before the rosin, so that the alum does the actual precipitating of the emulsified size.

Properties of finished board were found to be independent of the pre-cooking shredding treatment.

Semi-commercial cornstalk boards were produced which compared favorably with the best commercial boards in appearance, strength, insulating value, water resistance, resistance to humidity, expansion and general applicability.



# PRODUCTION OF YEAST GROWTH STIMULANTS BY MOLDS ON VARIOUS MEDIA<sup>1</sup>

H. H. SCHOPMEYER

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

It had been noticed in studying the growth of yeast in synthetic media that a considerably enhanced growth of yeast was obtained in those flasks which had been accidentally contaminated by molds. A systematic study of this phenomenon was undertaken, using several molds in different synthetic media. The molds employed were: *Aspergillus niger*, *Aspergillus clavatus*, *Trichoderma lignorum*, *Rhizopus nigricans* (plus and minus), and *Penicillium roqueforti*. The medium used for the growth of the yeast (*Saccharomyces cerevisiae*, No. 4226 Amer. Type Culture Collection) contained per 100 cc.: 0.188 gram of ammonium chloride, 0.10 gm. of dipotassium phosphate, and 10 gm. of sucrose. This is the Medium C of Fulmer, Nelson and Sherwood (1921). In the preliminary experiments two media were employed (Schopmeyer and Fulmer (1921)) for the growth of the mold, one identical with the medium described above, and the other containing per 100 cc., 0.05 gm. of magnesium sulfate, 0.01 gm. of dipotassium phosphate, 0.05 gm. of potassium chloride, 0.001 gm. of ferrous sulfate, 0.53 gm. of ammonium chloride and 10 gm. of glycerine. This medium is that developed by Naylor, Weisbrodt-Smith and Collins (1930) for the growth of *Penicillium roqueforti* except for the use of glycerol in the place of sucrose.

After the molds had grown for two weeks on these media at 25°, the mold felts were removed and the filtrates divided into two portions, one set being sterilized by steam and the other by filtration through Berkefeld filters. These media were tested for the presence of yeast growth stimulants. All the molds produced considerable quantities of the stimulants.

In order to study this phenomenon quantitatively, a synthetic medium was developed in order to obtain the best growth and chemical activity of the mold. This work was done using *Aspergillus niger*. The concentration of the components of the medium and pH were varied for optimum results. The growth was estimated by the weight of dry mold produced and the chemical activity by the titrable acidity developed. The best medium developed contained per 100 cc.: 0.07-0.1 gm. of magnesium sulfate, 0.10-0.15 gm. of dipotassium phosphate, 0.005-0.015 gm. of ferrous sulfate, 0.005-0.015 gm. of zinc sulfate, 1.75-2.25 gm. of ammonium chloride, and the appropriate substrate.

Using the appropriate media studies were made as to the nature of the extra-cellular and intra-cellular stimulant produced by the molds *Aspergillus niger* and *Aspergillus clavatus*. Glycerol was used as a substrate since the sucrose remaining in the medium rendered fractionation and purification difficult. Moreover, the use of pure glycerol removed the objection that the stimulants might be present in the sucrose as impurities.

---

<sup>1</sup>Original Thesis submitted December, 1931.

The stimulant is non-volatile and stable at boiling temperatures and is not appreciably affected by boiling for fifteen minutes with 10 per cent KOH or with HCl of equivalent strength. Eight liters of filtrate from a medium which had supported the growth of *Aspergillus niger* were evaporated to a small volume on a steam plate and then almost to complete dryness on a water bath. The resulting 50 gm. mass of dark gummy material was extracted with 95 per cent alcohol. The stimulant was found concentrated in the alcohol soluble fraction. Ether was added to the alcoholic solution to remove salts. About 7 gm. of precipitate were formed, 6 gm. remaining in the solution together with practically all of the stimulant. This material was boiled with norite to remove the coloring matter and evaporated. A clear sirupy material considerably more viscous than glycerol remained. About 1.5 gm. of this fraction were obtained and it represented practically all of the potency of the original filtrate. A 1 gm. sample of the purified material was dissolved in a very little water and 200 cc. of absolute alcohol were added. About 0.7 gm. of a granular material separated and upon evaporation 0.25 gm. of whitish crystalline material remained. The material insoluble in the alcohol contained practically all of the stimulant.

The material soluble in the ether alcohol solution was stable toward hot acid and hot alkali, which is not in accord with the properties of Bios as described by Wildiers (1901). The optimum concentration of this fraction was 80 mgm. per 100 cc., at this concentration the yeast crop was tenfold that obtained without the material. The stimulative materials produced by *Aspergillus niger* and *Aspergillus clavatus* are similar.

The properties of these stimulative materials were compared to those of Bios I and Bios II of Miller (Lucas, G. H. W. (1924), Eastcott, E. V. (1928)). The stimulants could not be separated into the fractions of Miller, nor did inositol enhance their effect. The stimulative materials were not identical with the bioses described by Fulmer, Nelson and Duecker (1924).

#### BIBLIOGRAPHY

EASTCOTT, E. V.

1928. The isolation and identification of Bios I. Jour. Phys. Chem., 32:1094-1111.

FULMER, NELSON AND DUECKER

1924. The multiple nature of Bios. Jour. Amer. Chem. Soc., 46:723-726.

FULMER, NELSON AND SHERWOOD

1921. The nutritional requirements of yeast. Jour. Amer. Chem. Soc., 43:186-191.

LUCAS, G. H. W.

1924. The fractionation of bios and comparison of bios with vitamins B and C. Jour. Phys. Chem., 28:1180.

NAYLOR, WEISBRODT-SMITH AND COLLINS

1930. Esterase and protease of *Penicillium roqueforti*. Iowa State College Jour. Sci., 4:465.

SCHOPMEYER AND FULMER

1931. Production of yeast growth stimulants by molds. Jour. Bact., 22:23-28.

# THE SOLUBILITY OF ROCK PHOSPHATE AS INFLUENCED BY SULFUR AND GYPSUM<sup>1</sup>

WINFIELD SCOTT

*From the Department of Farm Crops and Soils, Iowa State College*

Accepted for publication June 15, 1932

Experiments were carried out in the field and greenhouse to study the effects of elemental sulfur and of gypsum alone or with rock phosphate or rock phosphate and limestone, on crop growth, on various bacteriological activities and on the content of available phosphorus and other plant foods in the Waukesha silt loam and the Miami silt loam, two important soil types in Iowa.

The data secured from eight samplings on the Miami silt loam in the greenhouse test show that the nitrate content of the soil was depressed by the application of sulfur at the rate of 500 pounds per acre. Rock phosphate applied at the rate of one ton per acre and gypsum at the rate of 500 pounds per acre, alone or in combination, had little or no effect on the nitrate content of the soil at the different samplings. The limestone applied at the rate of one ton per acre stimulated nitrate production materially in this soil, but when rock phosphate was applied with the limestone, no greater effect was shown and when the sulfur or the gypsum was used with the limestone the nitrate content was reduced.

The sulfur treatment depressed the nitrifying power of the soil as measured by the tumbler method, using ammonium sulfate; tests being made at eight sampling dates. Gypsum had little effect on the nitrification process, sometimes showing slight increases and at other samplings having no effect. Rock phosphate when applied either alone or in combination had no influence on the nitrification process. Limestone brought about a distinct increase in the process.

The sulfate content of the variously treated soils at the different samplings was not influenced by any of the treatments except the sulfur and the gypsum, the former showing a pronounced and consistently stimulative effect.

The sulfofying power of the soils at the various samplings was determined by the tumbler method, adding sulfur, incubating two weeks and determining the sulfate content by the photometric method. The sulfur added to the soils in the greenhouse greatly increased the sulfofying power of the soils, according to the laboratory tests at the different samplings. Neither gypsum nor rock phosphate had any influence on the sulfofication process, but limestone brought about a very definite reduction in the process.

The amount of neutral ammonium citrate soluble phosphorus was determined in the soils at the various samplings and while the results varied somewhat it was evident that sulfur alone or in combination did increase the amount of available phosphorus in the soils. The gypsum and rock phosphate had no effect, but limestone brought about increases at most samplings. The limited effects of the treatments on available phosphorus in this soil is believed to be due to the low content of organic matter in the soil.

---

<sup>1</sup>Original Thesis submitted August, 1926.



The determinations of water-soluble phosphorus in the soils at the various samplings did not show any very definite effects of the treatments tested. Some increases were noted from the different fertilizers, but they were small and not of great significance.

The hydrogen ion concentration was determined colorimetrically at all samplings and it was found that sulfur increased the acidity or hydrogen ion concentration of the soil, whether it was applied alone or in combination with other fertilizers. Gypsum had no influence on the soil reaction and no effect was shown by the rock phosphate. The additions of limestone showed the usual normally large effects in reducing acidity.

The yields of soybeans on the Waukesha silt loam were secured for three years in the field tests and increases in crop yields were obtained from some of the treatments. The largest effects were shown by the treatment with rock phosphate plus limestone plus sulfur. In many cases the yields were so variable that conclusions were difficult to draw.

The yields of soybeans on the variously treated pots of Miami silt loam in the greenhouse showed more definite effects from the treatments. The sulfur reduced the yield in all cases whether used alone or in combination. The least depressive effect occurred when the sulfur was used with rock phosphate and limestone. Limestone had little or no effect on the soybean growth on this soil. Neither rock phosphate nor gypsum alone or in combination had any appreciable influence on the crop grown. The soil used in this work was only slightly acid, hence the limestone would not be expected to exert as great an effect as would be shown on a more acid soil. The sulfur treatment greatly reduced the nodule formation on the soybeans and this may be attributed to the increased acidity. This indicates that on more acid soils soybeans would be benefited to a much greater extent by limestone additions and that the crop would be better inoculated and hence more valuable from the protein standpoint.

The lack of effects from the rock phosphate and gypsum treatments on these soils from the crop standpoint and also from the bacteriological and chemical standpoints is quite striking and indicates that these natural materials may have little effect on some soils when applied as they were in these tests. Sulfur applied with rock phosphate may increase the availability of the phosphorus in the phosphate, but there is no large increase as might be expected from the increased acidity brought about by the sulfur. In fact, the limestone additions were also found to bring about some increases in the available phosphorus so that the reaction of the soil is evidently not the sole factor influencing phosphorus availability. In soils high in organic matter the effects of both the sulfur and the limestone might be quite different. Gypsum apparently does not affect the availability of the phosphorus in the rock phosphate.

A close correlation between the pH of the soils and the nitrifying power was shown, the latter increasing with treatments which decreased the hydrogen ion concentration of the soil. The influence of limestone in increasing the nitrifying power of the soils was definitely shown whether the limestone was applied alone or in combination. The depressing effect of sulfur treatments on nitrification was also correlated with increases in hydrogen ion concentration.

In general, it appears that no large nor striking effects of any of the treatments on the availability of the phosphorus in rock phosphate were brought about in these soils.



# BAND SPECTRA PRODUCED BY CERTAIN EXPLOSION MIXTURES<sup>1</sup>

HARLEY A. WILHELM

*From the Department of Chemistry, Iowa State College*

Accepted for publication June 15, 1932

This paper deals with the method of exciting molecular spectra by the combustion of mixtures of solids. Some observations on the spectra of MgS and PbS are given. A quantum analysis of a system of bands of MgS is included.

The work on molecular spectra was begun with the idea of studying emission spectra of intermetallic compounds. Failure to observe such spectra in the arc and spark between electrodes of the compounds led to a study of methods of exciting spectra of less metallic compounds. The aim of this part of the work was to proceed from these compounds to the more metallic compounds. In the study of excitation which followed, it was observed that flash-light powders produce band spectra. Out of this observation, the method of exciting band spectra by explosion mixtures has developed.

## THEORETICAL

When a change in electronic energy occurs in a gaseous diatomic molecule as it vibrates along the line joining the centers of the two nuclei and rotates on an axis perpendicular to this line, the total change in energy either by absorption or emission is

$$\Delta E = \Delta E_e + \Delta E_v + \Delta E_r.$$

$\Delta E_e$  is the energy change due to electron shift,  $\Delta E_v$  is the change connected with the change in vibrational state and  $\Delta E_r$  is that part of the change assigned to the change in rotational energy.  $\Delta E_r$  is small for the molecules studied in this work, and it will be neglected here since its effect is not resolvable on the spectrograph used.

From quantum mechanics,

$$\Delta E_v = hc[\omega_o'(v' + \frac{1}{2}) - \omega_o''x''(v'' + \frac{1}{2})^2] - hc[\omega_o''(v'' + \frac{1}{2}) - \omega_o''x''(v'' + \frac{1}{2})^2]$$

The (')'s refer to the upper electronic state while the (')'s refer to the lower electronic state. In this equation  $h$  is Planck's constant,  $\omega_o$  per second is the frequency of vibration of infinitesimal amplitude,  $v$  is the quantum number for the vibrational state and  $x$  is a small constant.

If the energy change is expressed as a wave number  $\nu$ , in ( $\text{cm}^{-1}$ ) units.

---

<sup>1</sup>Original Thesis submitted December, 1931.

$$\begin{aligned} \nu &= \nu_e + \nu_r \\ \nu &= \nu_e + [\omega'_0(\nu' + \frac{1}{2}) - \omega'_0 x'(\nu' + \frac{1}{2})^2] - \\ &\quad [\omega''_0(\nu'' + \frac{1}{2}) - \omega''_0 x''(\nu'' + \frac{1}{2})^2] \end{aligned}$$

All the constants and variables in this equation can be given values from the data by arranging the wave numbers for the bands of a system in a table for  $\nu'$   $\nu''$  assignments<sup>2</sup>.

#### APPARATUS AND MANIPULATIONS

The spectra were photographed with a Hilger El quartz spectograph. The Wratten and Wainwright panchromatic photographic plates which were used were sufficiently fast to register bands up to 6500 Å with very short exposures.

For general use, a one-fourth inch carbon rod having a cone shaped cup drilled in one end was used to support the charge. This rod was used in an upright position with the cup in the upper end and placed before a lens in such a way that the light from the reacting material was focused on the slit of the spectograph. To produce the MgS bands for final measurement, a magnesium rod was used to support the charge. This charge was a mixture of magnesium and magnesium sulfate.

In order to ignite the mixture an arc was struck on the side of the supporting rod in the vicinity of the cup. This method provided heat for the mixtures which required higher temperatures for their reactions to take place. The supporting rod was connected to the positive pole of a 100 volt storage battery.

Wave lengths of the band heads were determined by comparison with the iron arc spectrum. Wave lengths of the iron lines were plotted against micrometer readings on a large graph. The micrometer reading for a band head then gave from the graph the corresponding wave length in air.

The graininess of the photographic plate, which ordinarily makes weak lines very difficult to measure, was eliminated by an attachment which fits on the microscope of the Societie Genevoise measuring machine. This machine can be read to .0001 centimeter. The attachment, designed here, consists of a small metallic cube which rotates on one axis and has thin glass plates fastened over the two ends of each of two large holes which were drilled through the cube along the other two axes. The attachment is supported from the microscope and allows the small cube to rotate on an axis perpendicular to the axis of the microscope and between the object and the objective. The cube is housed and an adjustment, for turning the axis of rotation perpendicular to the lines on the plate, is provided for. A small aperture is left in the housing between the cube and the object. Over this aperture a small correcting lens may be placed, but such a lens was not necessary with the magnification of the microscope used in this work. As the cube is rotated the image of each grain travels in one direction across the field. With a speed of four or five revolutions per second the grains which make up a spectrum line are fused into a solid, well defined line. The field can be easily examined for spots by stopping the rotating cube. The cube can be rotated by a belt from a small motor to a pulley on the axis shaft extended outside the housing.

<sup>2</sup>Meggers and Wheeler, B. S. Jour. Research, 6, 239 (1931).

A Monroe calculating machine was used to carry out the calculations in the work.

### EXPERIMENTAL

In the course of the work it was observed that a flash-light powder made of magnesium and barium peroxide showed the BaO bands. The PbO and MnO bands were found in the spectra produced by mixtures of magnesium with lead dioxide and manganese dioxide, respectively. When other substances such as sulfur were added to the mixtures new bands appeared in their spectra.

In the work, following the above observations, certain advantages and disadvantages of the method of exciting molecular spectra by combustion mixtures have been found.

The advantages of this method as compared with some other methods are:

(a) The intensities of the atomic spectra are very small when compared to the intensities of the molecular spectra produced by explosion mixtures.

(b) Systems are excited which are difficult to excite otherwise.

(c) A small amount of equipment is necessary.

The disadvantages so far encountered in using the mixtures are:

(a) The spectra are weak in the ultra-violet.

(b) A weak continuous background may appear in the spectrum.

(c) The large number of elements involved in the combustion of a mixture makes the selection of the carrier of a system less certain.

(d) The time necessary to photograph a spectrum is long unless very fast photographic plates are used.

The work was narrowed for the present to the excitation of spectra of the sulfides of the metals. By the method of elimination, used to find the carrier of a band spectrum produced by the combustion of a mixture, spectra have been assigned to MgS, PbS and CuS.

### RESULTS

Several bands throughout the blue and violet have been shown to be due to MgS. These bands make up more than one system. A system, which is well isolated in the blue and made up of bands which degrade to the red, has been measured and the wave numbers set up in a table for  $v'$   $v''$  assignments. From this table, the approximate equation

$$v = 23,055.8 + [495.3(v' + \frac{1}{2}) - 2.8(v' + \frac{1}{2})^2] - [525.2(v'' + \frac{1}{2}) - 2.93(v'' + \frac{1}{2})^2]$$

has been worked out to represent the system.

This system is produced by an electron shift between two levels sep-

arated by 2.85 volts. If the  $v-\omega_v$  graph<sup>4</sup> is asumed to give a straight line to  $\omega_v = 0$ , the energy of dissociation in the lower electronic level is about 67,000 g.-cal per g.-mole.

A spectrum consisting of more than 100 bands between 6600 and 4100 Å is produced during the combustion of a mixture of aluminum, lead dioxide and sulfur. These bands degrade to the red and show no fine structure in the dispersion used. The spectrum resembles the PbO spectrum<sup>3</sup>, but the two when photographed through a Hartmann diaphragm show that the mixture produces bands which with other evidences can be assigned to PbS. A quantum vitbrational analysis for these bands has not yet been completed.

Bands carried by CuS have been observed in the yellow region of the spectrum. They make up about five sequences which degrade to the red. The spectrum has been excited by the copper are in an atmosphere of sulfur vapor, and by the explosion of a mixture of magnesium and copper sulfate. Further measurements and work are to be done on this spectrum.

#### SUMMARY

1. The method of exciting band spectra by explosion mixtures has wide applicability.
2. The advantages of the explosion method outweigh the disadvantages in the production of many molecular spectra.
3. The spectra of some metallic sulfides have been excited by the combustion of mixtures.
4. A quantum vibrational analysis has been given for one band system carried by MgS.

---

<sup>3</sup>Bloomenthal, Phys. Rev., **35**, 34 (1930).

<sup>4</sup>Birge and Sponer, Phys. Rev., **28**, 259 (1926).



## Author Index

- Allen, Edward S., 251  
 Aquino, Dionisio I., 65  
 Baker, Merle Porter, 409  
 Becker, Elery R., 131, 299  
 Bergman, H. D., 227  
 Brown, F. E., 133  
 Brown, Robert E., 11  
 Buchanan, J. H., 367  
 Burtner, Robert R., 389  
 Carter, James Hal, 413  
 Coles, Harold W., 33, 43  
 Cook, Wendell Burnham, 417  
 Coons, Robert Roy, 419  
 Drake, C. J., 347  
 Dickey, Joseph B., 137, 381  
 Edgar, Rachel, 395  
 Erwin, A. T., 277  
 Fang, Hsi Ch'on, 423  
 Farrar, Milton D., 325  
 Gilman, Henry, 11, 133, 137, 381, 389  
 Gilman, Joseph C., 357  
 Haas, Louise E., 287  
 Hager, Anna, 299  
 Hall, Phoebe R., 131, 299  
 Hammer, B. W., 89  
 Harris, H. M., 347  
 Harris, Stanton A., 425  
 Hartford, Charles Earl, 429  
 Harwood, H. James, 431  
 Henderson, Everette Lee, 435  
 Hewitt, E. A., 143, 227  
 Hewlett, Amiot P., 137, 439  
 Hussong, R. V., 89  
 Hyde, Lincoln Spencer, 447  
 Irwin, Margaret House, 451  
 Kendall, Sara E., 17  
 Lounsberry, C. C., 277  
 Martin, J. N., 277  
 Michaelian, Michael B., 455  
 Mills, Harlow B., 263  
 McNeely, J. K., 1  
 Oglesby, W. T., 227  
 Patrick, Roger, 457  
 Peet, Louise Jenison, 463  
 Porter, R. H., 95  
 Quig, Joseph Bradley, 467  
 Richardson, Charles H., 287  
 Richardson, Roger W., 469  
 Schopmeyer, H. H., 471  
 Scott, Winfield, 473  
 Shilling, E. W., 1  
 Shull, W. Earl, 325  
 Shumaker, John B., 367  
 Tate, H. D., 347  
 Travis, Bernard, 317  
 Van Peurse, Ralph L., 133  
 Werkman, C. H., 17  
 Wilhelm, Harley A., 475  
 Winton, Eleanor, 395  
 Yeager, J. Franklin, 325

## Subject Index

- Abnormal reactions of organometallic compounds, 425  
 Abstracts of doctoral theses, 407  
 Accuracy of dilution method of estimating the density of population of micro-organisms, 251  
 Acetaldehyde, 460  
 Acetylene, 5  
 Acid bacteria, propionic classification of, 17, nomenclature of, 17  
 Acidophilus milk, 450  
 Acids volatile, formation of, 455  
 Action of a transverse electrostatic field upon flames, 1  
*Aerobacillus acetoethylicus*, cultural characteristics of, 460  
*A. asterosporus*, cultural characteristics of, 460  
*Aerobacillus*, fermentation of levulose by bacteria of genus, 457  
*A. polymyxa*, 460  
 Agricultural wastes, 429  
 Albino rat, lactation in, 463  
 Alcohol, furfuryl, 15, 133  
 Alkylation, by means of sulfonic esters, 11  
 organomagnesium halides, 11  
 Amines, halogenated tertiary, 427  
 Amerosporium, host for parasite fungi, 357  
 Antiseptics, 441  
 Aphididae, agents in transmission of virus diseases, 347, 348  
 Aphids as vectors of yellow dwarf, 347  
*Aphis rumicis*, Linn., 353  
 Aqueous solutions of butanol, ethanol and acetone, 424  
*Aquilegia canadensis*, 96, 97  
*Architomocerura crassicauda* Denis, 263, 265  
*Asclepias syriaca*, 97, 99, 104  
*Aspergillus clavatus*, use of, 471, 472  
 Aspergillus, host for parasitic fungi, 357  
*Aspergillus niger*, use of, 471, 472  
 Atomic spectra, intensities of, 477  
 Bacteria, method for estimating number in unit volume, 251  
 Bacteria, propionic acid, 17  
 classification and nomenclature, 17  
*Bacterium acidi propionici a*, 17  
*B. acidi propionici b*, 17  
*Bacillus acidi propionici*, 17  
*B. calidolactis*, 89  
 action of, in milk, 90

- in evaporated milk, 91
- description of, 92
- observations on, 89
- Band spectra produced by certain explosion mixtures, 475
- Barium peroxide, use of, in preparation of per acids and their salts, 420
- Benzene, 5
- Benzoyl furan and *p*-thiocresol, 386
- Benzylmagnesium chloride, 13
- Biometric studies on *Eimeria*, 299
- Blood, coagulation of, 325, 328
- Blood counting, 235
- Blood constituents, determination of, 172
- Blood filtrates, 146
- literature on, 146
- Blood of cholera-infected swine, 175, 232, 239
  - erythrocytes in, 232
  - hemoglobin content of, 232
  - leucocytes in, 233
- Blood of farm animals, chemical composition of, 162
- Blood of laboratory animals, composition of, 158
- Blood of normal and cholera-infected swine, comparisons of, 243
- Blood of normal swine, 173, 238, 239
  - calcium in, 191
  - erythrocytes in, 227
  - hemoglobin content of, 229
  - inorganic phosphorus, 189
  - leucocytes in, 229
  - non-protein nitrogen in, 176
  - pre-formed creatinine, 183
  - sugar, 187
  - urea nitrogen in, 178
  - uric acid in, 181
- Blood samples, 235
  - procedures for analysis of, 170
- Blood sugar, 152
- Butter cultures, relationships among organisms in, 409
- Calcium carbonate, addition to milk for keeping qualities, 410
- Calcium content in blood, 156
- Calcium carbonate, addition of, to milk, 410
- Capsicum, floral structures related to nectaries in genus, 277
- Capsicum, nectaries of, 277
- Caramel color, study of, 367
- Caramelan, 367
- Caramels, colloidal properties of, 375
  - effect of cold acids on, 378
  - preparation of, 367
  - preparation of, at different temperatures, 372
  - stability of, 380
- Carbinols, secondary and tertiary, preparation of, 443
- Carbohydrates, fermentation of, by species of *Propionibacterium*, 19
- Cellular coagulum, 336
- Cementites, stability of, 437
- Characteristics of *Eimeria miyairii* and *E. separata*, 300
- Chemical and morphologic phases of blood of normal and cholera-infected swine, 143, 227
  - concentration of certain chemical constituents, 143
  - certain morphologic phases, 227
- Chenopodium murale* L., 104
- Cholera-infected swine, blood of, 175
  - calcium in, 201
  - creatinine in, 198
  - creatine plus creatinine, 199
  - inorganic phosphorus, 200
  - non-protein nitrogen in, 194
  - sugar in, 200
  - urea nitrogen in, 195
  - uric acid in, 197
- Chloride, furfuryl, 389, 390
- Chloride, 5-methyl-2-furfuryl, 389
  - 5-nitro-2-furfuryl, 389
- Chlorides of silicon, preparation of, 467
- Citrullus vulgaris* Schrad., 108
- Cholera-infected swine, blood of, 143, 227
- Chromium, 435, 437
- Cladotrichum, host for parasitic fungi, 357
- Cinnamyl chloride, reaction of, with magnesium, 425
- Citric and lactic acids, formation of volatile acids from, 455
- Coagulation of blood, preparation of smears, 328
  - process, 326
- Coagulation of blood from cockroach, 325
- Coccidia, effect of, on health of rat, 311
- Coccidium, new species of, from Norway rat, 131
- Cockroach, coagulation of blood from, 325
- Collembola, new and rare North American, 263
- Colloidal properties of caramels, 375
- Conjugated systems in furan types, 381
- Cornstalks, in development of synthetic lumber, 469
- Corpuscles, volume percentage of, 238
- Corrosion of galvanized sheet iron, 414
- Cost data on design of plant for production of insulation board, 429
- Creatinine and creatine, 150
- Cucumbers, classification of varieties, 111
  - hybridization of, 111, 112
  - mosaic, 100
  - types of, 103
- Cucumis anguria*, 108, 116
- sativus*, 112, 116
- sativus*, var. *anglicus*, 111
- Cucurbita pepo*, 104
- Cystinyl peptides and guanidine derivatives, physiological action of, 431, 432
- Derivatives of furfural, 439

- Design of a plant for the production of insulation board from agricultural wastes and cost data on this process, 429
- Development of synthetic lumber from cornstalks, 429
- Di-alkyl glucoses, 38, 39
- Dietary factors affecting lactation in albino rat, 463
- Dilution method of estimating the density of a population of micro-organisms, 251
- Discussion of synonymy in the nomenclature of certain insect flagellates, with the description of a new flagellate from the larvae of *Ligyroides relictus* Say (Coleoptera Scarabaeidae), 317
- Effects of molybdenum and chromium on the malleabilization of white cast iron, 435
- Electric heater, construction of, 369
- Electric wind, 7
- Electrical precipitation of suspended matter, bibliography of, 8
- Electrolytic method, in preparation of per acids and their salts, 420
- Electrostatic field and electron theory, bibliography of, 8
- Electrostatic field, transverse, 1
- Entomobrya intonsa* n. sp., 263, 265
- Entomobrya nigriceps*, n. sp., 263, 268
- Entomobrya triangularis* Schött, 263, 269
- Epemys norvegicus*, species of *Coccidium* from, 131
- E. miyairii*, and *E. separata*, patent period for, 302
- quantitative, biometric and host-parasite studies on, 299
- E. miyairii*, number of oocysts produced during infections with, 308
- E. separata*, a new species of *Coccidium* from the Norway rat (*Epemys norvegicus*), 131
- E. separata*, number of oocysts produced during infections with, 309
- Esters, sulfonic, 11
- Erythrocytes, 227
- Ethylene, 3
- Ethyl furylacrylate and *p*-thiocresol, 388
- Explosion mixtures, band spectra produced by, 477
- Fermentation of levulose by some bacteria of the genus *Aerobacillus*, 457
- Ferrosilicon, 468
- First supplementary list of parasitic fungi from Iowa, 357
- Floral structures in genus *Capsicum*, 277
- Fluorine method, in preparation of per acids and their salts, 421, 422
- Fungi, parasitic from Iowa, 357
- Furan compounds, 11
- Furan derivatives, physical properties of, 137
- lachrymatory, 140-141
- sternutatory, 140, 141
- vesicant, 140, 141
- Furan series, dyes of, 443
- insecticides of, 441
- lachrymators of, 441
- Furan types, conjugated systems in, 381
- Furfural diacetate, 441
- Furfural and some of its derivatives, 439
- Furfural and *p*-thiocresylmagnesium iodide, 386
- Furfural and benzoyl furan with *p*-toluenesulfonic acid, 386
- Furfural and *p*-thiocresol, 386
- Furfural derivatives, 137
- Furfural, hydrogenation of, 133
- separation of produces, 133
- Furylacrylic acid and *p*-thiocresol, 388
- Furylacrylic acid and *p*-toluenesulfonic acid, 388
- Furfuryl alcohol, identification of, 133
- Furfuryl chloride, 12, 390
- Furyl-alkyl groups, introduction of, 11
- Furylacrylic esters, 137
- Galvanized sheet iron, corrosion of, 414
- Gas, production of
- hydrogen, 2
- ethylene, 2, 3
- methyl alcohol, 3
- methane, 3
- Gasoline, 5
- Glomerularia, host for parasitic fungi, 357
- Glucose and sucrose, sterilization of solutions of, 417
- Glucosazone, 34
- Glucose derivatives, 33, 43
- mono- and di-alkylated, 33
- tri-, tetra-, and penta-alkylated, 43
- Glucose,
- pentamethyl, 58
- trimethyl, 43, 44
- Glucoses, tetra-alkylated, 52
- Graphitization of white cast iron, 435
- Grignard reagents from halogenated tertiary amines, 427
- Guanidine derivatives, physiological action of cystinyl peptides and, 431
- Halides, 12
- organomagnesium, 15
- tetrahydrofurfuryl, 14
- Halogenated tertiary amines, Grignard reagents from, 427
- Hemoglobin, 229
- Hemoglobin determinations, 238
- Heterocycles, in furan types, 381
- Hexamastix confusa* n. nom., 319
- Host parasite studies on *Eimeria*, 299
- Hosts, differential, for cucumber virus, 108
- Hosts for parasitic fungi in Iowa, 361
- Host-specificity studies, 312
- Hybridization experiments with cucumbers, 111
- Hydrocarbons, combustion of, 1



- Hydrocarbon flame, 7  
 Hydrogenation of furfural, 133, 136  
 Hydrogen,  
   flame in electrostatic field, 7  
   gas, production of, 2  
   molecule, 7  
 Hydrogen peroxide method in preparation  
   of per acids and their soils, 420  
 Hydrogen sulphide, 7
- Infections of *Eimeria miyairii*, number of  
   oocysts produced, 308  
 Immunity of coccidian infections, 312  
 Insecticides of furan series, 441  
 Insulation board, design of plant for pro-  
   duction of, 429  
 Integration, method of, 261  
 Introduction of furyl-alkyl groups by  
   means of sulfonic esters, 11  
 Iron-copper system, 413  
 Isosaccharosan, formation of, 374  
 Lachrymators of furan series, 441  
 Lactation in albino rat, dietary factors af-  
   fecting, 463  
 Lactobacilli, study of, 447  
*Lactobacillus acidophilus*, 450  
*Lactobacillus bulgaricus*, 447  
*L. casei*, 447  
 Leucocytes, 229, 233  
   classification of, 236  
 Levulose, fermentation of, 457  
 Ligrodes larvae, 319  
 Lower chlorides of silicon, preparation of,  
   467  
 Literature of alkylated carbohydrates, 33,  
   43  
 Lumber, synthetic, 469  
*Lycium barbarum*, nectary in, 279  
 Malleabilization of white cast iron, effects  
   of molybdenum and chromium on, 435  
 Mercaptans, 137  
 Methane, 3  
 Methyl alcohol, 3  
 Methyl ether, 5-nitro-2-furfuryl, 389  
 5-Methyl-2-furfuryl chloride, 389  
 2-Methyl glucose, 33  
 Methyl saccharic acid, 34  
 Metallic sulfides, spectra of, 478  
 Micro-organisms, dilution method of esti-  
   mating density of population of, 251  
 Molybdenum, 435, 437  
 Mono-alkylated glucose derivatives, 33  
 Mono-alkyl glucoses, 33, 34, 35, 36, 37  
 Monocercomonas Grassi, 317  
*Monocercomonoides ligrodis* n. sp., 319  
*Monocercomonoides* n. nom., 318  
 Mono-furfuryl phthalate, 134  
 Mosaic, reactions of cucumbers to, 95, 102  
   in backcrosses of cucumbers, 113  
   source of virus of, 99  
*Myzus persicae*, 104
- Nectar, chemical study of, 278  
   secretion of, 277
- Nectaries of Capsicum, 277  
 New and rare North American Collembola,  
   263  
 Nicotine, relative toxicity of, 287, 291, 296  
 5-Nitro-2-furfuryl acetate, 392  
 5-Nitrofurfuryl alcohol, oxidation of, 393  
 5-Nitro-2-furfuryl chloride and 5-nitro-2-  
   furfuryl methyl ether, 389, 391, 392  
 5-Nitrofurfuryl chloride and sodium  
   methylate, 393  
 Nitrogen, sulfur and, of wool, 395  
 Non-protein nitrogen, 147  
 Normal and cholera-infected blood, com-  
   parison of, 202  
 Normal swine, blood of, 143, 227  
 Normal swine, blood of, 173  
   total non-protein nitrogen in, 176  
 Norway rat, new species of *Coccidium*  
   from, 131  
 Nuclear nitro groups, 389
- Observations on *Bacillus calidolactis*, 89  
 On the coagulation of blood from the cock-  
   roach *Periplaneta orientalis* (Linn.).  
   with special reference to blood smears,  
   325  
 Oocysts, counting of, 301  
   measurements of, 301, 302  
 Organisms in butter cultures, 409  
 Organometallic compounds, abnormal reac-  
   tions of, 425  
*Oryzaephilus surinamensis* (L.), 296  
 Osazone, 34  
 Oxonium compound, 388  
 Oxidation, sulfur, 65
- Parasitic fungi from Iowa, supplementary  
   list of, 357  
 Pentamethyl glucose, 58  
 Per acids and their salts, preparation of,  
   419  
*Periplaneta orientalis* (Linn.),  
   coagulation of blood from, 325  
 Phlytaena, host for parasitic fungi, 357  
 Phosphorus, compounds in blood, 154  
*Physalis alkekengi* L., 101, 278  
 Physiological action of cystinyl peptides  
   and guanidine derivatives, 431  
 Physiological properties of some furan de-  
   rivatives, 137  
*Plodia interpunctella*, 296  
 Preliminary experiments with aphids as  
   vectors of yellow dwarf, 347  
 Preparation of per acids and their salts,  
   419  
 Preparation of secondary and tertiary  
   furyl carbinols, 443  
 Preparation of the lower chlorides of sili-  
   con, 467  
 Probable error in estimating number of  
   micro-organisms in unit volume, 252, 262  
 Production of insulation board, plant for,  
   429



- Production of yeast growth stimulants by molds on various media, 471
- Product of factors of type, 253
- Propane, 1, 5
- Propionic acid bacteria, classification and nomenclature, 17
- Propionibacterium, Orla-Jensen, 1909, generic diagnosis, 22
- Propionibacterium Freudenreichii* van Niel, 18  
description of, 23  
*P. Jensenii* van Niel, 18, 24  
*P. pentosaceum* van Niel, 18, 24  
*P. Peterssonii* van Niel, 18  
description of, 23  
*P. raffinosaceum*, 20  
sp. nov., description of, 30  
*P. rubrum*, van Niel, 18  
description of, 29  
*P. Shermanii* van Niel 18  
description of, 25  
*P. technicum* van Niel, 18  
description of, 28  
*P. Thönii* van Niel, 18  
description of, 27
- Propionibacterium, species names of, 18  
genus, 21  
key to, 20
- Protozoa, method of estimating number in unit volume, 251
- Pseudosinella folsomi* Denis, 272  
*P. pettersoni* Börner, 272  
*P. rolfsi* n. sp., 263, 272  
*P. violenta* Folsom, 272
- Pyridine and nicotine, relative toxicity of, 287, 291, 296
- Pyridine and nicotine, vapor concentration of, 290
- Pyrrole, preparation of, from ammonium xylonate, 423
- Quantitative, biometric and host-parasite studies on *Eimeria miyairii* and *E. separata* in rats, 299
- Rat, albino, lactation in, 463
- Rat, as host for species of coccidia, 299
- Rat, effect of coccidia on, 311
- Reactions, abnormal, of organometallic compounds, 425
- Reaction of cucumbers to types of mosaic, 95
- Relationships among the organisms in butter cultures, 409
- Relative toxicity of pyridine and nicotine in the gaseous condition to *Tribolium confusum* Duval, 287
- Rhinotrichum, host for parasitic fungi, 357
- Rhynchosporium, host for parasitic fungi, 357
- Rock phosphate, solubility of, 473
- Rumex crispus* L., 104
- Salina banksi* MacGillivray, 276  
*S. decorata* n. sp., 263, 273, 276  
*S. trilobata* Schött, 276
- Secreting mechanism in genus *Capsicum*, 279, 280
- Silicon, lower chlorides of, 467
- Sodium peroxide method, preparation of per acids and their salts, 421
- Solanum dulcamara*, 278
- Some dietary factors affecting lactation in the albino rat, 463
- Some of the relationships among the organisms in butter cultures, 409
- Sphaerita* sp., 320
- Solanum melongena* L., 101
- Solubility of rock phosphate as influenced by sulfur and gypsum, 473
- Stalizing effect of nuclear nitro groups in furan types, 389
- Standard deviations, 259, 261
- Steenbock growth ration, 300
- Streptococcus citrovorus*, 455  
*S. lactis*, 409  
*S. paracitrovorus*, 455
- Stimulants for yeast growth, by molds, 471
- Studies on sulfur oxidation, 65
- Sterilization of solutions of glucose and sucrose, 417
- Study of some of the *Lactobacilli*, 447
- Studies in vitamin A technic, 451
- Studies on sterilization of solutions of glucose and sucrose, 417
- Study of utilization of xylose, 423
- Study of caramel color, 467
- Study of corrosion of galvanized sheet iron, 414
- Suaeda moquini* Greene, 104
- Sucrose, 370
- Sucrose, and glucose, sterilization of solutions of, 417
- Sulfonic esters, 11
- Sulfur and nitrogen of wool, 395
- Sulfur oxidation, 65  
in relation to bacterial activity, 66  
soil activity, 67  
studies on plot soils, 68  
laboratory studies on, 77
- Sulfur oxidizing efficiency of soils, 83
- Swine blood, cholera infected, 239, 241
- Swine, blood of normal, 173  
blood of cholera-infected, 175  
creatine plus creatinine, 186  
calcium in, 191  
inorganic phosphorus in, 189  
non-protein nitrogen in, 176  
preformed creatinine in, 183  
sugar in, 187  
urea nitrogen in, 178  
uric acid in, 181
- Swine blood, normal, 238, 239
- Synthetic lumber, development of, from cornstalks, 469

- Tetra-alkylated glucoses, 52  
Tetrahydro-furyl-alkyl groups, 11, 12  
Tetrahydrofurfuryl halides, preparation of, 14  
Tetrahydrofurfuryl-phenyl-methane, 12  
Tetrahydrofurfuryl iodide, 14, 15  
Tetrahydrofurfuryl *p*-toluenesulfonate, 13, 15  
Theses, abstracts of doctoral, 407  
*p*-Toluenesulfonic acid, 11  
  chloride, 13, 15  
Transverse electrostatic field, action of, 1  
Tri-alkylated glucoses, 43  
*Tribolium confusum* Duval, relative toxicity of pyridine and nicotine in gaseous condition to, 287, 291  
Trimethyl glucose, 43, 45, 46  
*Tullbergia iowensis* n. sp., 263, 264  
Turpentine, 7  
*Tyroglyphus longior*, 296  
Urea, in blood, 147  
Uric acid, 149  
Utilization of xylose, 423  
  
Vectors of yellow dwarf, 347  
Vitamin A depletion period, 452, 454  
Vitamin A free basal diet, 451  
Vitamin A technic, 451  
  
Vitamin A test animals, standardization of, 454  
Virus, cucumber, 96, 105, 107  
  mosaic, 96  
  "white pickle," 96  
Volatile acids formed by *Streptococcus* sp., 455  
Volatile acids formed from citric and lactic acids by *Streptococcus citrovorus* and *S. paracitrovorus*, 455  
  
Wool, analysis of, 400, 401  
Wool, sulfur and nitrogen of, 395  
White cast iron, 435  
"White pickle mosaic," resistance of varieties of cucumber plants to, 97, 98  
  inoculation trials, 97  
  response of plants to, 103  
*Willemia denisi* n. sp., 263, 264  
  
X-ray investigation of the iron-copper system, 413  
Xylose, fermentation of, 423  
  oxidation of, to xylonic acid, 423  
  study of utilization of, 423  
  
Yeast growth stimulants, on media, 471  
Yellow dwarf, aphids as vectors of, 347